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TITLE: Identification of Autoantibodies to Breast Cancer Antigens in Breast Cancer Patients

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**14. ABSTRACT**
The purpose of this project is to identify breast cancer antigens and autologous antibodies that breast cancer patients make to those antigens. The scope of this project is the development of new targeted therapeutics to treat breast cancer. The major findings of this project are the methods developed for successful extraction of mRNA from breast tumor specimens and synthesis of a cDNA library that can be expressed as protein fragments on the surface of phage. Future plans include identifying tumor associated antigens via binding of protein fragments to antibodies found in patient serum and characterization of the antibodies identified. The identification of new breast cancer antigens and characterization and replication of autologous antibodies in the laboratory using phage display represent significant and novel information for the diagnosis and treatment of breast cancer.

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None provided.

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## Table of Contents



## Introduction

The purpose of this research is to identify breast cancer specific protein fragments from a patient and identify autologous antibodies that bind to those fragments. cDNA libraries have been created from tumors of patients with breast cancer to identify antigenic proteins for those individuals using a modification of the SEREX technique. The scope of this project is to develop a method to utilize a patient's own immune system to make targeted therapeutics. Therefore, scFv libraries representing the immune repertoire from those same patients will be created and tested against the earlier identified antigenic proteins. Binding scFvs can then be evaluated as a therapeutic target. In addition, the project will identify autoantibodies that may serve as biomarkers with potential use in breast cancer diagnosis.

## Body

An update on progress for the previous year is described in Table 1.

**Table 1. Summary of Overall Project**

| Phase | Task | Statement of Work Projected Completion | Status |
|---|---|---|---|
| 1 | 1 | Months 1-6 | Complete |
| | 2 | Months 6-12 | In progress |
| 2 | 3 | Months 12-16 | In progress |
| | 4 | Months 16-20 | Not initiated |
| | 5 | Months 20-34 | Not initiated |

Phase One. Generation of breast cancer phage displayed library and identification of autologous antibodies to phage displayed breast cancer proteins.

Task One. Create cDNA phage library from a patient with breast cancer.

Within the past year, standardized protocol for collection of sample procurement has been developed that allows the cancer tissue from a single patient to be used both as a source of high quality RNA and tissue for subsequent histological analysis of tumor-binding antibodies. To our knowledge, no other research group uses this method. The use of core biopsy punches ensures pathological evaluation of the gross cancer specimen for clinical purposes and accomplishes research aims including

quantification of tumor volume (calculated from the dimensions of the core biopsy device), rapid tissue acquisition to prevent RNA degradation, and acquisition of several separate tumor pieces for subsequent histological analysis.

Briefly, breast cancer surgical specimens for this study are quickly transported from the operating room to pathology. In pathology, the specimen is weighed, inked for margins and divided into 1cm sections. The dimensions of the tumor are documented and gross margin evaluation performed. Upon completion of these clinical tasks, a disposable cylindrical skin punch biopsy device is used to obtain several 1cm long cores of tissue with a diameter of 2mm. For example, a 1cm tumor will allow 10 to 14 good quality cored samples. The cored specimens are flash frozen and then stored at -80C.

To isolate RNA, tissues are thoroughly homogenized in Trizol with a Polytron homogenizer. RNA is extracted from Trizol, precipitated in ethanol, and further purified with a QIAGEN RNeasy kit. See Appendix slides 21 and 115 for examples of tumor RNA isolated using this method. The samples in slide 115 demonstrate better quality associated with standardization of our methods.

Last year we reported the construction of cDNA libraries using breast cancer cell lines and de-identified tumor RNA, but were unable to express the library properly in phage (Appendix Slide 23). Since then several cDNA libraries were constructed using the SMART cDNA Library Construction kit (Clontech). This kit has advantages over the lambda ZAP expression system used previously. cDNA synthesis with the SMART method requires 1/500$^{th}$ of the source material, does not require mRNA purification, produces protein fragments in three reading frames, and ensures that the entire length of mRNA is converted to double stranded cDNA. As a result, these libraries are a better representation of the repertoire of protein fragments found in a patient's tumor and still achieve both the objective and anticipated outcome of Task 1.

This new method was first tested using breast cancer cell line (SK-BR-3) RNA to create first and second strand cDNA (Appendix Slide 25), purified to remove primers and cDNA less than 500bp in length, ligated into the lambdaTriplEx vector (Clontech) and packaged into phage (Gigapack packaging extracts, Stratagene). By February 2010 (15 months after the project began), this library was titered, amplified, and titered again (Appendix Slide 118 titers were initially miscalculated, please see Table 1 for correct calculations). The initial library contained 1.63 x10$^6$ clones. We checked this library for quality with blue-white selection and found that greater than 90% of clones contained recombinant insert. We plan to do random individual clone analysis of this library in the future.

**Table 2. Description of SMART cDNA expression libraries created using various sources of RNA.**

| Library | Source | Size | cDNA insertion rate |
|---|---|---|---|
| SK-BR-3 | breast cancer cell line | 1.63x10$^6$ | > 90% |
| 188-6 | fresh frozen tumor | 1.08x10$^6$ | 86-96% |
| hPlacental | purchased, Clontech | 4.80x10$^6$ | 80% |
| 09-067-1 | fresh frozen tumor | 5.71x10$^6$ | 54% |

Following production of this library, standardized protocols were created for the procurement of tissue and blood and production of a cDNA library, and patients were recruited into the study for specimen collection. By September 2010, four sets of tissue samples and blood have been collected for this study

and are currently being processed. The RNA from the first patient sample has served as source material for a cDNA library synthesized using the SMART construction kit and packaged into lambda phage. cDNA analysis in Figure 1 demonstrates several libraries from source RNA with first strand cDNA varying from 0 to 10kB in length. Table 1 contains the data for highest titer from control (hPlacental RNA provided by Clontech) and breast tumor libraries.

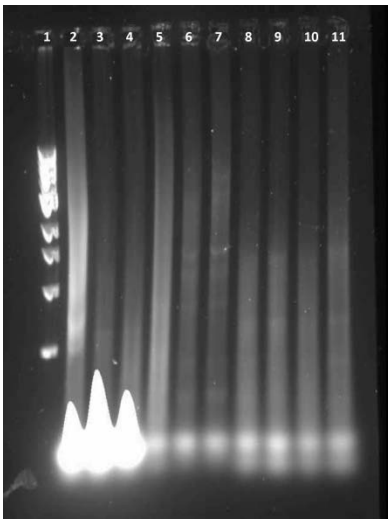



**Figure 1. Agarose gel electrophoresis of cDNA libraries. Lane 1: molecular weight ladder (ranging from 0.5 to 10kB), Lane 2: hPlacental cDNA (control), Lanes 3-11 libraries constructed from patient tumor (09-067-1)**

During the construction of this library (April – May 2010), the size fractionation process used to purify cDNA was evaluated to reduce the amount of time for library creation and maximize cDNA quantity for downstream use. Briefly, several methods for removing primers and short length cDNA from the library were compared including size fractionation columns from Clontech and Invitrogen and molecular weight filtration devices (Microcon and Nanosep). Despite a sizeable loss of cDNA, the size fractionation column from Clontech yielded the most consistent results with the best quality cDNA.

The remaining tumor samples will be processed in the next month and at least five more sample sets will be collected over the next year. Since the new protocol for library construction is fully developed from tissue procurement to expression library titering, the amount of time it takes to build one library has been reduced to one week.

Outcome:  A cDNA expression library from a breast cancer patient

Task Two. Select phage displayed breast cancer antigens that bind to immobilized antibodies from the same breast cancer patient's serum.

In May 2010, the first patient library was synthesized and ready for screening against autologous serum (Appendix Slide 118). During this time, I discovered that the proposed method for selecting breast cancer antigens would not work because the library was not displayed on the surface of lambda phage, but rather expressed in bacterial colonies. As a result, screening with beads was not possible with this library because the cDNA and proteins were not physically linked as with phage display. Since the technique proposed in the original Statement of Work was not scientifically possible with a lambda expression library, a modification of the SEREX technique [1] was developed for application in this project. This technique still accomplishes the same objective using a different screening modality.

Briefly, the library is plated at a density of 10,000 pfu/150mm plate with isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce the expression of breast cancer protein fragments. A

nitrocellulose membrane soaked in IPTG is laid on the agar plate and incubated for several hours at 37C and then lifted off the plate. The membrane is washed in TBS with 0.05% Tween several times, blocked with 5% nonfat milk in TBS, washed several more times with TBS, and incubated overnight with 1:100 subtracted [2] autologous patient serum in TBS. The next day, the membranes are washed again with TBS-Tween and then incubated with a secondary antibody (goat anti-human IgG, Jackson Immunoresearch). Following several more washes with TBS, the membranes are developed with SIGMAFAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets (Sigma). This protocol is clearly more time consuming than phage display, as a library of over a million clones is going to require processing at least 100 membranes over weeks compared to a day. However, this method has the added benefit of a better quality cDNA library to work with, so theoretically positive clones are more likely to be true positives.

In July 2010, this library was screened with autologous serum with no apparent positive clones. Troubleshooting and backtracking led to the conclusion that the library did not contain recombinant inserts due to two mistakes. First, a critical step (restriction digestion) in the process was inadvertently missed. Second, blue-white selection was not completed on the library before it was screened, therefore the fact that it lacked inserts was not evident. However, the advantage to this “test run” is that a protocol for screening was developed and valuable knowledge gained troubleshooting each step in the process. Since this time, several cDNA libraries were synthesized from patient samples with special attention to purification and ligation steps as well as evaluating the library once it is expressed in bacteria (See table 2 for most recent data on library from patient 09-067-1). I anticipate screening a new library in October 2010.

Anticipated Outcome: isolation and identification of breast cancer protein fragments (deliverable) to which patients have pre-existing antibodies.

Phase Two. *Ex vivo* identification and amplification of patient antibodies that bind to breast cancer protein fragments

Task Three. Generate phage display antibody library from the patient’s B-cells.

Last year no progress was reported on this task. Since that time, several sources of B cells from patients with breast cancer were obtained to generate antibody libraries. In addition to the tumor tissues (see Task 1), blood and sentinel node scrapings were collected from four patients with breast cancer.

In the past year, protocols were developed for obtaining these tissues on the day of surgery. Blood is collected from the patient in the operating room and brought back to the lab for processing. Peripheral blood mononuclear cells (including B cells) are isolated using a Ficoll-PAQUE PLUS gradient and frozen for later extraction of RNA. With this sample, RNA is extracted in a similar manner to that described in Task 1.

Obtaining tumor draining sentinel node scrapings proved more difficult because most of the lymph node is saved for diagnostic procedures in pathology. Therefore, Donald Weaver, a pathologist specializing in breast cancer, was consulted to identify a strategy to obtain research source material while preserving the integrity of the node for pathological analysis. Briefly, a sterile scalpel is used to scrape the bivalved lymph node when it is checked intra-operatively for the presence of breast cancer metastasis. The scrapings are transported in sterile PBS with an RNase inhibitor (Superase-in, Ambion) promptly to the laboratory on ice. The cells are pelleted and RNA is extracted using methods similar to those described in Task 1 (homogenization is not necessary) (Appendix Slide 75).

Also, preliminary work has begun on this task by creating several scFv libraries in the laboratory using

source material other than lymph nodes. A member of our laboratory, Stephanie Pero, has generated multiple scFv libraries using bone marrow and blood. These are the same methods that will be used to make libraries from the sentinel nodes. Briefly, the cDNA was synthesized from 30 µg of total RNA using the Superscript III first-strand synthesis system (Invitrogen) with a combination of random hexamers and oligodT primers to ensure broad representation of antibody classes. To avoid, as far as possible, a bias in antibody variable chain family representation, each V family of variable regions (VH or VL) was amplified by independent PCR, with a total of 45 different reactions according to previously published methods [3]. A PCR-overlapping process was performed in order to join both V domains. The DNA segments encoding the assembled products were fused to the pIII gene of the pComb3XSS phagemid vector [4]. The scFv library has good diversity and is comprised of $1.38x10^7$ members. Ninety-six independent colonies were sequenced and all the clones included both the heavy and light chains. Using V-base DNA Plot software (http://www.vbase2.org), we found the variable regions were distributed across the full repertoire of antibody germ line genes.

In addition, I was accepted into a course on Phage Display at Cold Spring Harbor Laboratories that will take place in October 2010. While there, I will create a scFv library with experts in the field to familiarize myself with the methodology utilized in Task 3.

I anticipate completion of Task 3 for at least one patient's source material in the next six months.

Anticipated Outcome: three phage display antibody libraries per patient derived from B-cells recovered from the blood, lymph nodes, and tumor

Tasks four and five in phase two of the statement of work are not yet complete.

Task Four. Select phage displayed antibodies that bind to selected breast cancer proteins

Task Five. Determine sensitivity and specificity of individual phage antibody clones for binding to breast cancer.

## Key Research Accomplishments

- It was observed that higher quality RNA could be obtained from tumors by using biopsy punches and an acceptably short time from surgical resection to flash freezing tumor samples. Proper tissue homogenization also increased RNA quality.
- Much less source material was needed to produce cDNA libraries using SMART methods than with the lambda ZAP construction kit to produce cDNA that ranged from 0.5 to 3kB in length and a library than contained over a million clones.
- Size fractionation produced better quality cDNA while reducing overall yields as compared to other size fractionation columns and molecular weight filters. Size fractionated libraries had a higher percentage of recombinant clones and more clones overall than other methods tested despite having a lower yield of cDNA.
- Four lambda expression libraries containing over a million different clones each were synthesized from various sources of cell line and tissue RNA. More than 90% of clones from lambda expression libraries derived from SK-BR-3 breast cancer cell line and breast tumor cDNA contained proper inserts.
- High quality RNA was obtained from patient lymph node scrapings by using RNAse inhibitors when during procurement while not compromising patient material needed for clinical use.

## Reportable Outcomes

Presentations

"Characterization of Patient Autoantibodies to Breast Cancer Using Phage Display" Cell and Molecular Biology Program Seminar. December 15, 2009.

"Tumor Autoantibodies: Potential Therapeutic or Irrelevant Phenomenon?" Surgical Research Seminar. May 12, 2010.

"A high molecular weight melanoma-associated antigen-specific chimeric antigen receptor redirects lymphocytes to target human melanomas" MD/PhD Journal Club. May 13, 2010.

"Characterization of tumor associated antigens using a recombinant expression of a patient derived breast cancer cDNA library and autologous serum" MD/PhD Research Day. July 23, 2010.

## Conclusions

In this summary, completion of task one and progress on tasks two and three of this project have been presented. A unique standard protocol was developed to obtain tumor and other specimens from patients that ensured high quality RNA and extra material for histological testing. A cDNA expression library was synthesized from the tumor of a patient with breast cancer using a method that selects for full-length cDNA and considerably less source material than traditionally used in the SEREX process. Success with this modification translates into the ability to screen libraries derived from much smaller tumors (i.e. less than 1cm) or even core biopsy samples taken before the patient has surgery. Libraries synthesized will soon be tested against autologous serum and positive clones evaluated, using a modified protocol that is similar to the SEREX technique. Positive clones may provide information to the scientific and medical communities as tumor-associated antigens and may be of use as biomarkers for diagnostics or for use in therapeutics such as cancer vaccines or adoptive immunotherapies. For this project, however, they will serve as targets in the isolation and identification of binding scFv derived from the same patient. Development of scFv libraries is already in progress and will continue to be created and evaluated for quality as targets are being isolated in task three. Isolation and identification of binding scFv to targets derived from autologous patient tumors represents a customized approach to breast cancer therapy that has the potential to reduce side effects associated with systemic therapy and resistance observed with many current targeted therapies.

## References


1. Sahin, U., et al., *Human neoplasms elicit multiple specific immune responses in the autologous host.* Proc Natl Acad Sci U S A, 1995. **92**(25): p. 11810-3.
2. Tureci, O., et al., *Identification of tumor-associated autoantigens with SEREX.* Methods Mol Med, 2005. **109**: p. 137-54.
3. Andris-Widhope, J., et al., *Generation of Antibody Libraries: PCR Amplification and Assembly of Ligh- and Heavy-chain Coding Sequences*, in *Phage Display: A Laboratory Manual*, B. Barbas, Scott, Silverman, Editor. 2001, Cole Spring Harbor Laborary Press: Cold Spring Harbor. p. 9.1-9.35.
4. Scott, J.K. and C.F. Barbas, *Phage-display Vectors*, in *Phage Display: A Laboratory Manual*, C.F. Barbas, et al., Editors. 2001, Cold Spring Harbor Laboratory Press: New York. p. 2.1-2.19.

## Appendix

Appendix Slides 1-39. Presentation from December 15, 2009
Appendix Slides 40-77. Presentation from May 12, 2010.
Appendix Slides 78-108. Presentation from May 13, 2010.
Appendix 109-123. Presentation from July 23, 2010.

# Characterization of Patient Autoantibodies to Breast Cancer Using Phage Display

Leah Novinger
Krag Laboratory
CMB Seminar
December 15, 2009

# Presentation Overview

- Background
  - Tumor Immunology
  - Targeted Therapy
- Methods
  - SEREX
- Results
  - cDNA Library Synthesis
- Conclusions and Future Research

# The Humoral Immune Response to Cancer

A beneficial immune response to cancer cells exists, but may not be enough to eliminate the tumor

"The presence of tumor infiltrating lymphocytes is associated with better prognosis in individual patients" (Jäger 2007)

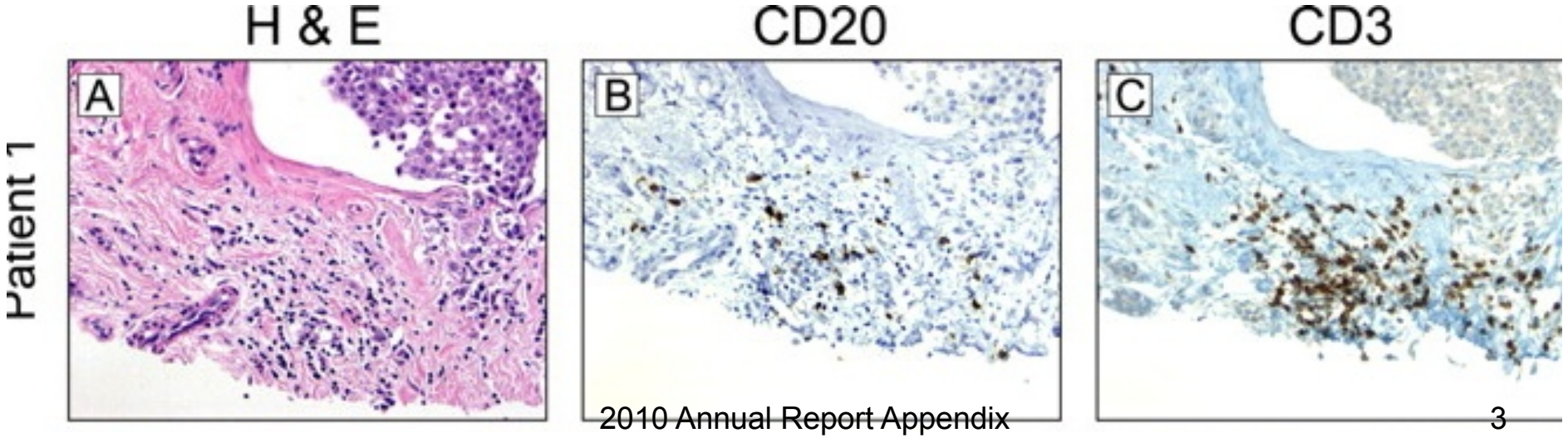




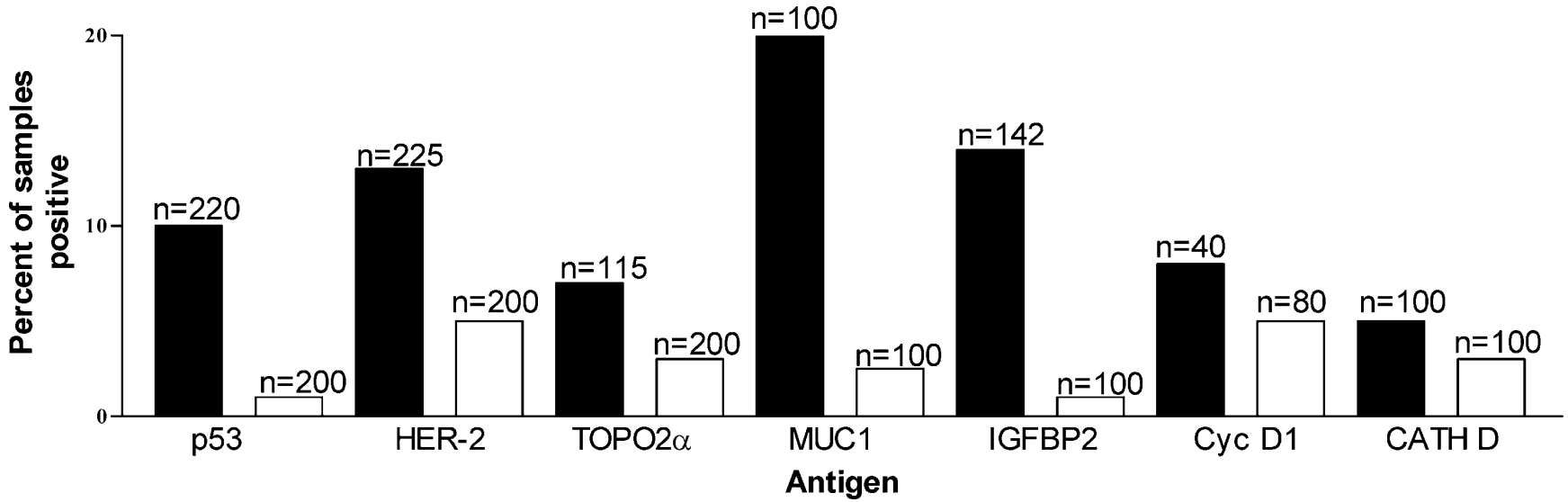

Image source: Coronella et al. J Immunol. 2002 Aug 15;169(4):1829-36.

# The Humoral Immune Response to Cancer

- Tumor autoantibodies can be detected in sera
  - Sometimes before cancer diagnosis (Lubin 1995, Li 2005)
  - ~50% breast cancer patients (Cornella-Wood, 2003)
    - Her-2/neu antibodies
- The antibody response may be associated with different subsets of disease
  - ER/PR+ in breast cancer

# Autoantibodies as Biomarkers

Antibodies to tumor associated antigens are more frequently detected in sera from breast cancer patients than from normal donors




Shown are the percentages of individuals positive for serum antibody to 7 tumor antigens. Gray columns show the response in patients; white columns show the response in control normal donors. The number of patients or controls tested for each antigen were indicated at the top of the column.



Source: Hailing Lu; Vivian Goodell; Mary L. Disis; *J. Proteome Res.* **2008,** 7, 1388-1394.

# Data correlating antibody response to prognosis are conflicting

- Robust B cell response may be associated with tumor progression (Disis 2009)
- Best responses are associated with early disease (Cornella-Wood 2003)
- Epitope spreading and cross priming associated with a better response to therapy (JCO 2002 and Knuts JCI 2001)
- Immunoreactivity to MDA-MB-231 lysates was correlated with worse prognosis (Hamsher et al 2007)
- Immune response to MUC1 antigen in early breast cancer is correlated with better survival (von Mensdorff-Pouilly 2000)

# Targeted Therapeutics

- Are drugs or other substances that **block** the growth and spread of cancer **by interfering with specific molecules** involved in tumor growth and progression (NCI Fact Sheet)

- Potential Functions:
  - Disrupt signaling pathways necessary for growth or proliferation
  - Promote apoptosis
  - Initiate an immune response
  - Deliver substances toxic to the cell

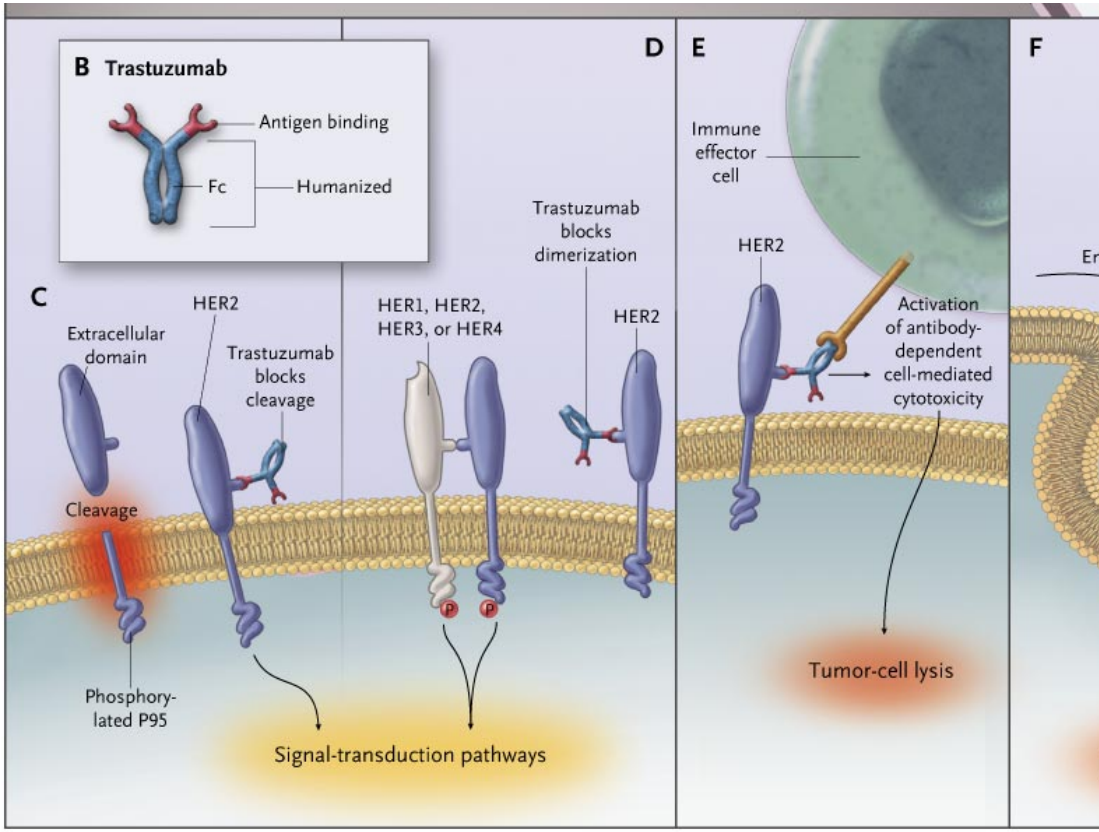
- Targeted therapies for breast cancer patients can reduce resistance to treatments
  - Resistance occurs eventually in most patients due to genetic amplification (Gonzales-Angulo 2007)

# Examples of Targeted Therapy

Trastuzumab (Herceptin®) binds HER-2 on breast cancer cells

Gefitinib (Iressa®) binds Epidermal Growth Factor Receptor on lung cancer cells

Temsirolimus (Torisel®) inhibits mTOR kinase activity in renal cell carcinoma

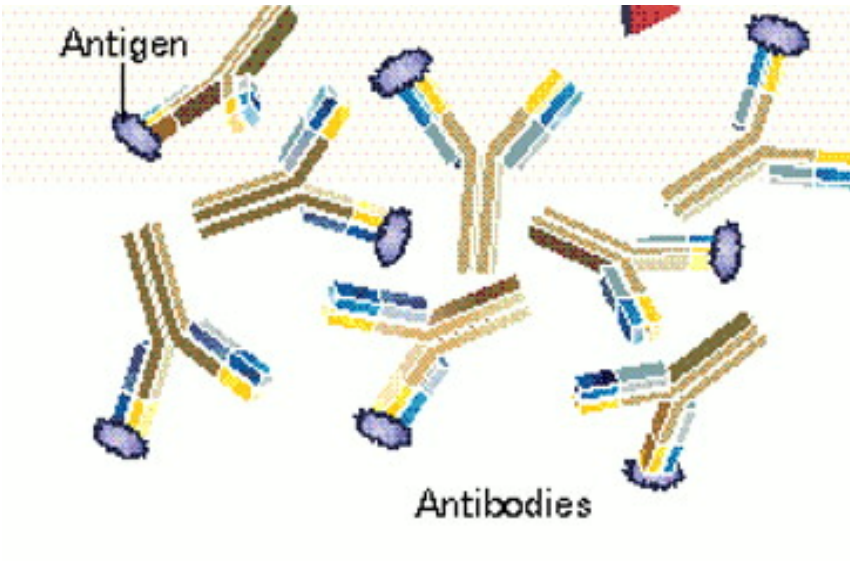





Potential Mechanisms of Action of Trastuzumab Source: Hudis C. N Engl J Med 2007;357:39-51

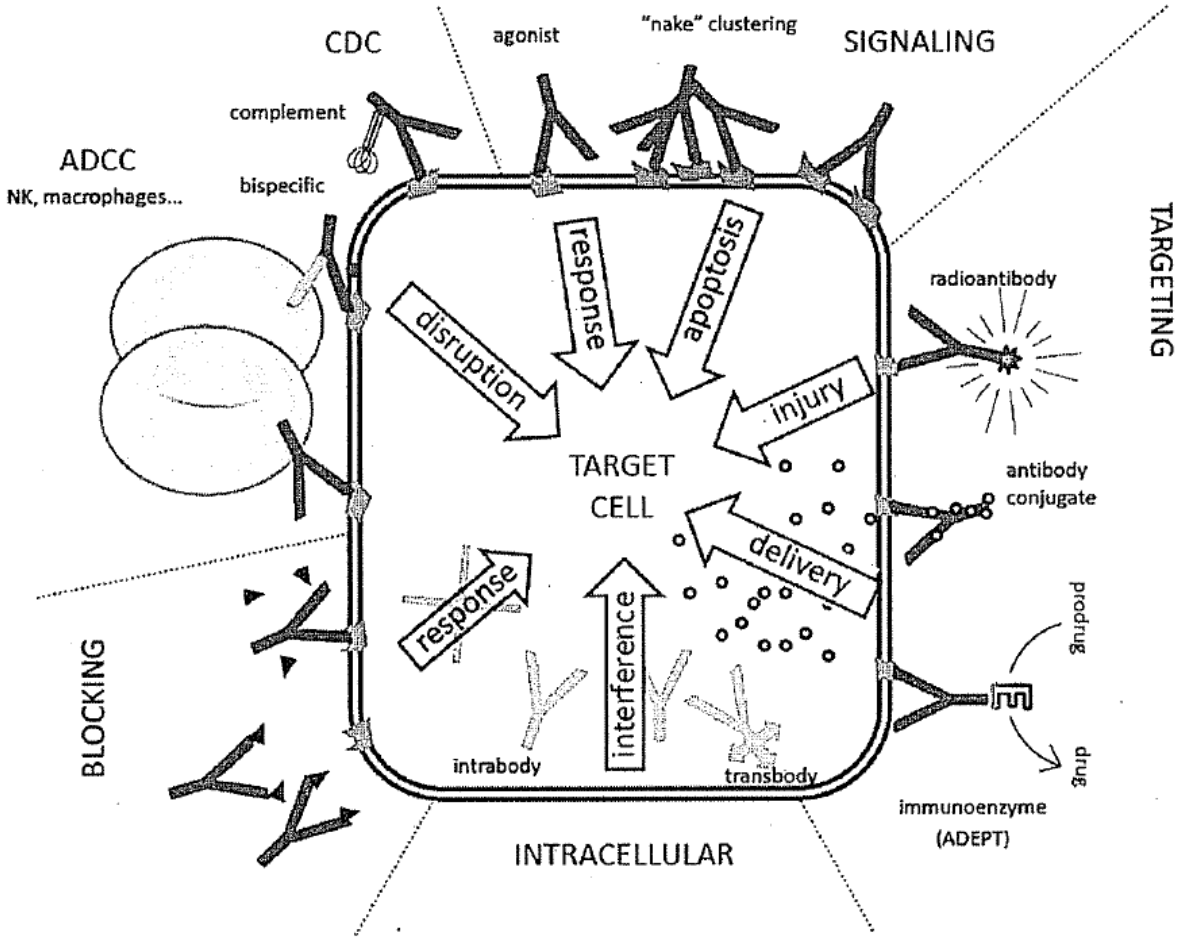
# Criteria for Antibody Use

1. Bioactivity
2. Affinity
3. Specificity




Target Validation

# Bioactivity

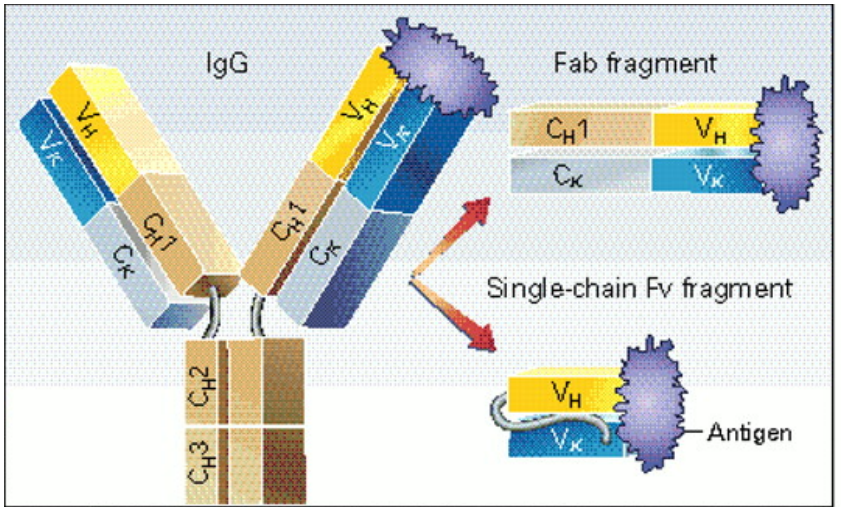





Source: Nieri P Donadio E Rossi S Adinolfi B Podestà A . Curr Med Chem 2009 ;16(6):753-79

# Specificity and Affinity

- Cancer antigens identification is achieved through the SEREX technique
  - Cancer-testis
  - Over-expressed
- The source of a single change variable fragment library (scFv) is the patient antibodies against the tumor




Source: **Marks C and Marks J. N Engl J Med 1996;335:730-733**

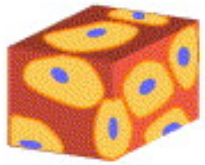
# Phage Display

# SEREX is a technique developed to identify the tumor associated antigens using patient autoantibodies

1. A cDNA expression library is created from a tumor and inserted into lambda phage
2. The phage library is panned on antibodies that came from a cancer patient
3. Protein fragments to which the patient has developed antibodies can then be identified and characterized

# Modified SEREX

1. Tumour tissue





Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

# Hypothesis

Patients develop anti-tumor antibodies that can be identified, isolated, and reproduced for therapeutic purposes

# Research Questions

What kind of antigens are expressed on the patient's tumor?

What is the antibody profile against tumor associated antigens in different tissues?

# Project Overview

Aim 1: Generate a phage displayed cDNA expression library representative of tumor antigens

Aim 2: Identify peptides from the library to which patient antibodies bind

Aim 3: Create scFv libraries from different sources of tissue in the same patient and identify tumor binding scFv antibodies

# Project Overview

Aim 1: Generate a phage displayed cDNA expression library representative of tumor antigens

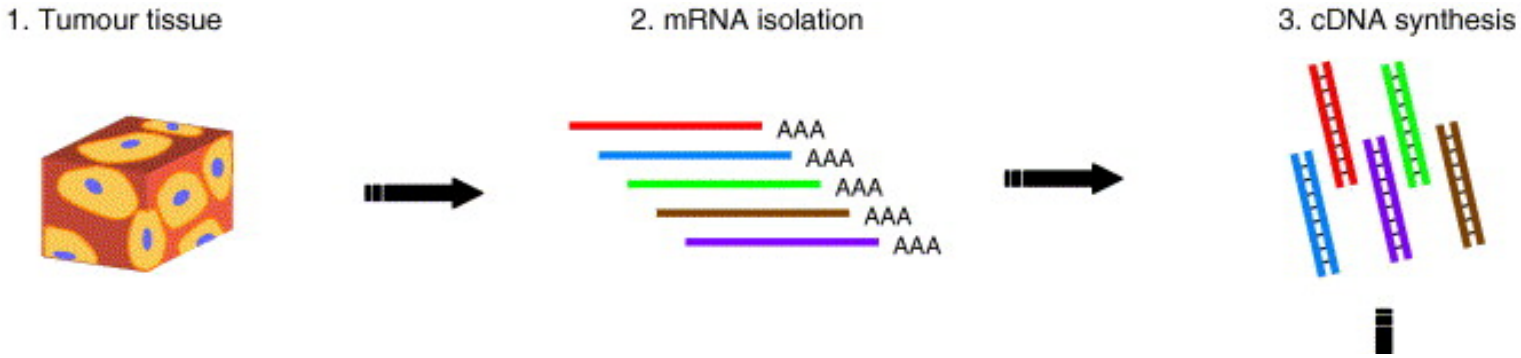
Aim 2: Identify peptides from the library to which patient antibodies bind

Aim 3: Create scFv libraries from different sources of tissue in the same patient and identify binding scFv antibodies

# Generate a phage displayed cDNA expression library

- Why is it more useful than microarray?
- What are the advantages and drawbacks?
- What is the expected outcome?

# Modified SEREX

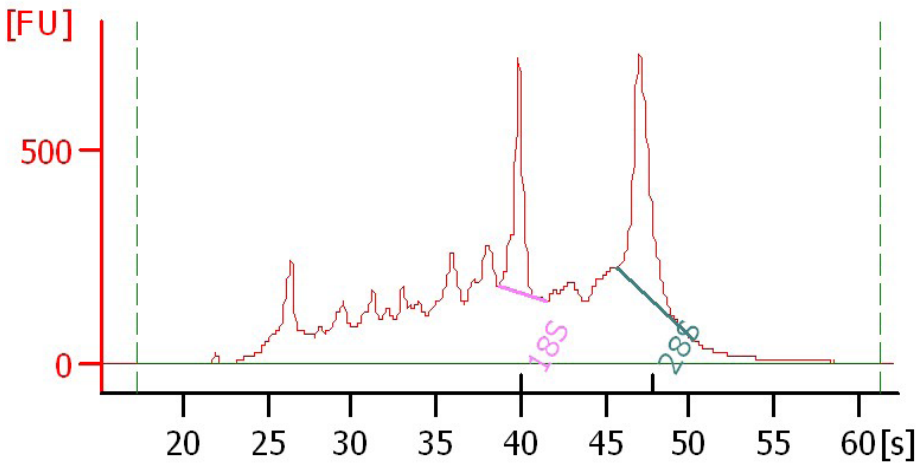




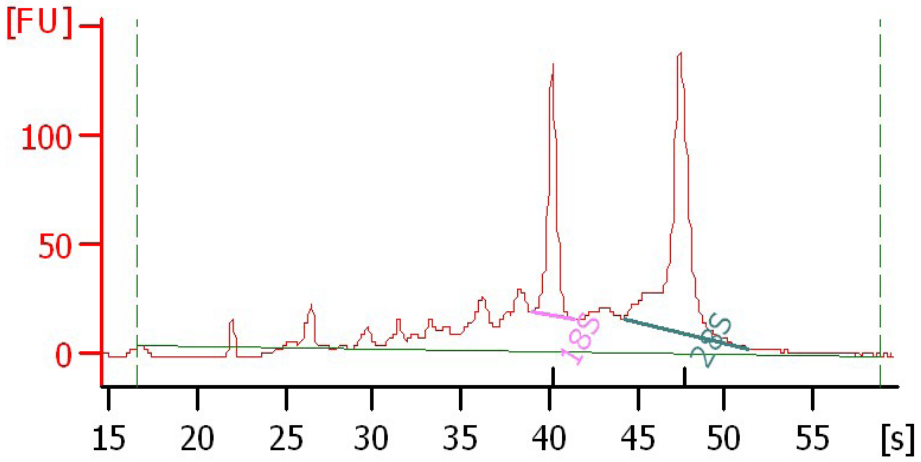

Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

# Tumor Tissue RNA Isolation

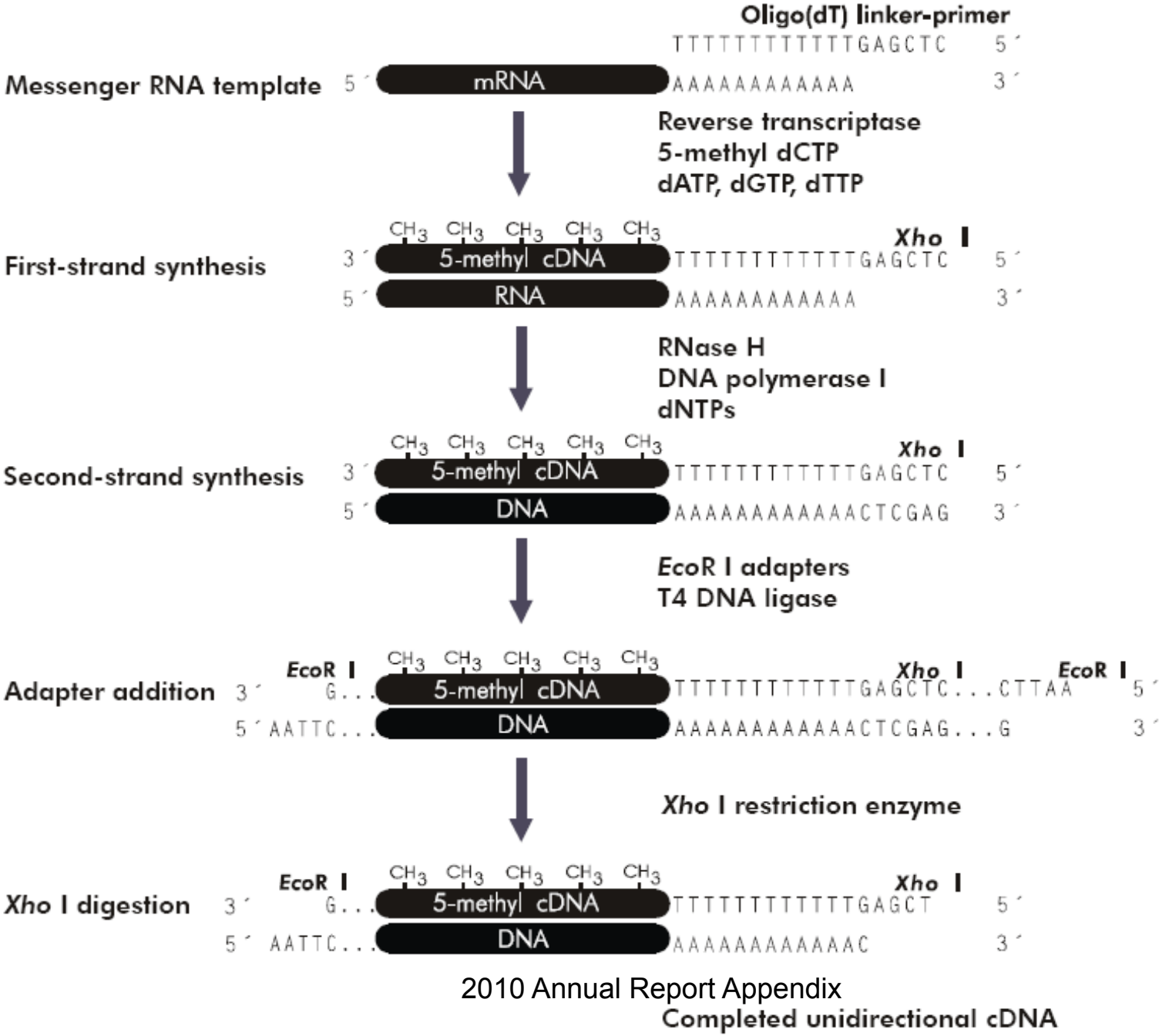
A




B




Deidentified breast carcinoma tissue was obtained from FAHC Surgical Pathology and flash frozen. Samples were stored at -80C. On the day of the experiment, samples were removed from the freezer and transferred to a conical tube with several ml of Trizol and homogenzed with a polytron homogenizer. Following RNA isolation, samples were evaluated with an Agilent Bioanalyzer. Results in A and B are representative samples and demonstrate two rRNA peaks with slightly more degradation than seen with SkBr3 cells.


Oligo(dT) linker-primer
TTTTTTTTTTTTGAGCTC 5´
Messenger RNA template
5´ mRNA AAAAAAAAAAAA 3´
Reverse transcriptase
5-methyl dCTP
dATP, dGTP, dTTP
First-strand synthesis
CH3 CH3 CH3 CH3 CH3
Xho I
3´ 5-methyl cDNA TTTTTTTTTTTTGAGCTC 5´
5´ RNA AAAAAAAAAAAA 3´
RNase H
DNA polymerase I
dNTPs
Second-strand synthesis
CH3 CH3 CH3 CH3 CH3
Xho I
3´ 5-methyl cDNA TTTTTTTTTTTTGAGCTC 5´
5´ DNA AAAAAAAAAAAACTCGAG 3´
EcoR I adapters
T4 DNA ligase
Adapter addition
EcoR I
CH3 CH3 CH3 CH3 CH3
Xho I
EcoR I
3´ G... 5-methyl cDNA TTTTTTTTTTTTGAGCTC...CTTAA 5´
5´AATTC... DNA AAAAAAAAAAAACTCGAG...G 3´
Xho I restriction enzyme
Xho I digestion
EcoR I
CH3 CH3 CH3 CH3 CH3
Xho I
3´ G... 5-methyl cDNA TTTTTTTTTTTTGAGCT 5´
5´ AATTC... DNA AAAAAAAAAAAAC 3´
Completed unidirectional cDNA

# cDNA Library Synthesis

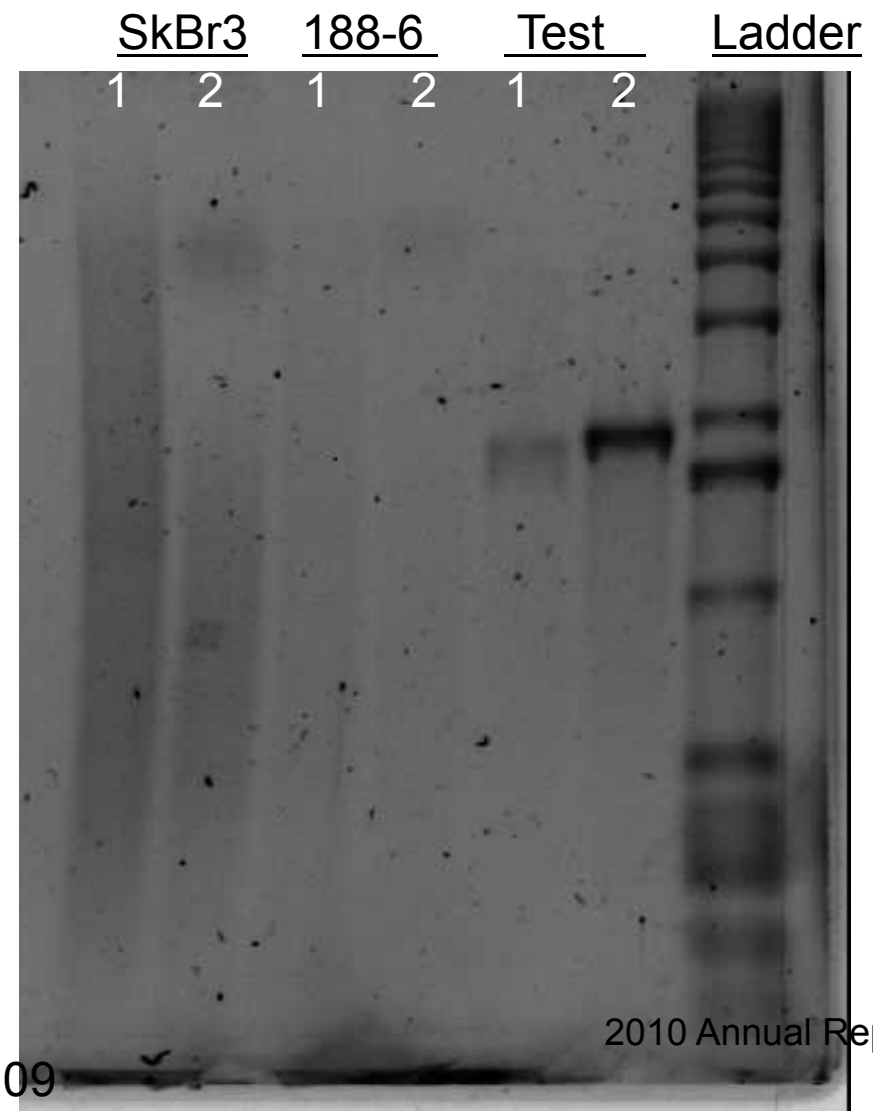
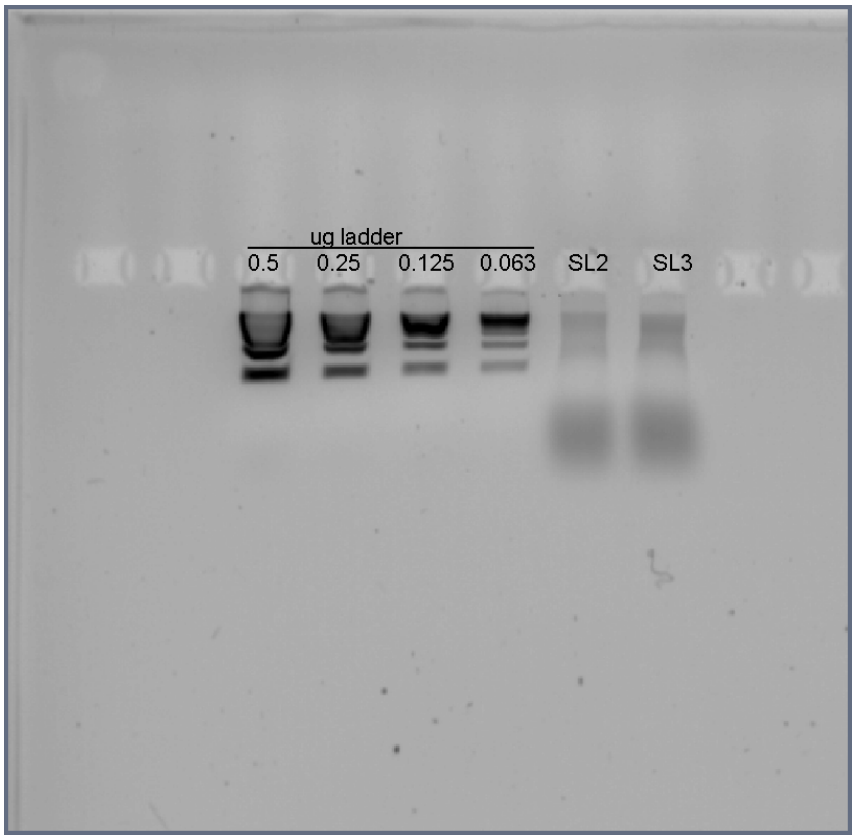



Figure 9. cDNA was synthesized from 2-5ug mRNA from Skbr3 cells, breast carcinoma tissue (188-6) or Test RNA (all 1.6kB) provided by Stratagene. First and second strand synthesis reactions were evaluated by alkaline agarose electrophoresis, which denatures bound strands but retains secondary structure that may have inhibited synthesis reactions. A ladder ranging from 1-10kB was used in the experiment. A dark smear was observed for both first and second strand SkBr3 synthesis reaction. A lighter but similar looking smear was observed for 188-6 samples (de-identified breast cancer tissue). An isolated band was observed for Test RNA, consistent with expected results since all mRNA was the same length. No high or low molecular weight bands were observed, which would be indicative of “hairpinning”, or folding of the RNA or cDNA that would have prevented a proper synthesis reaction from occurring.

# Ladder Titration

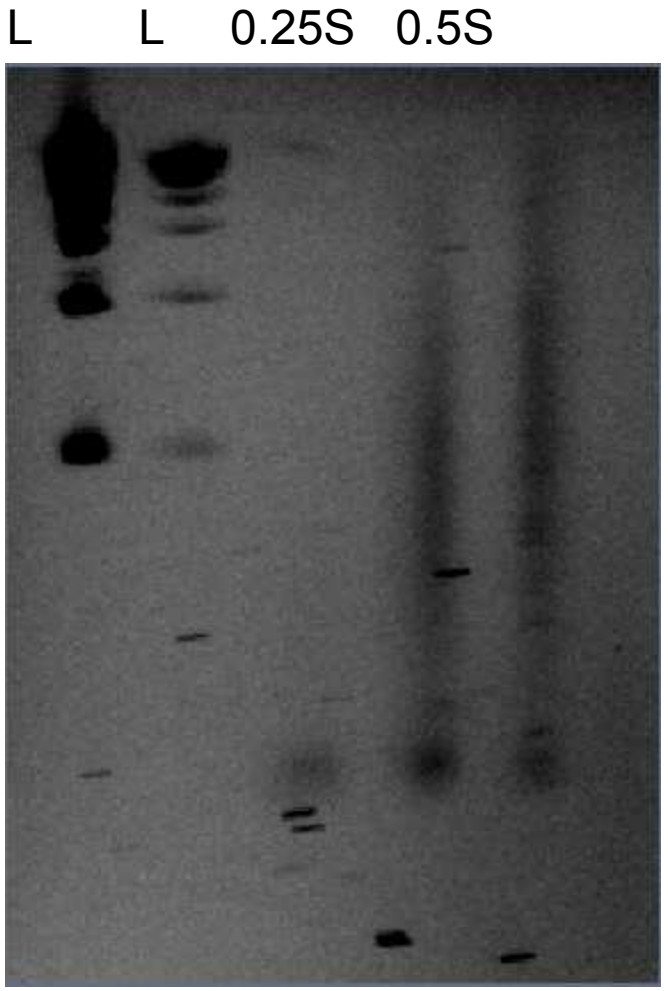
Based on findings both libraries probably contain far less than 500ng of cDNA and synthesis reaction was not complete

Quantity became an issue

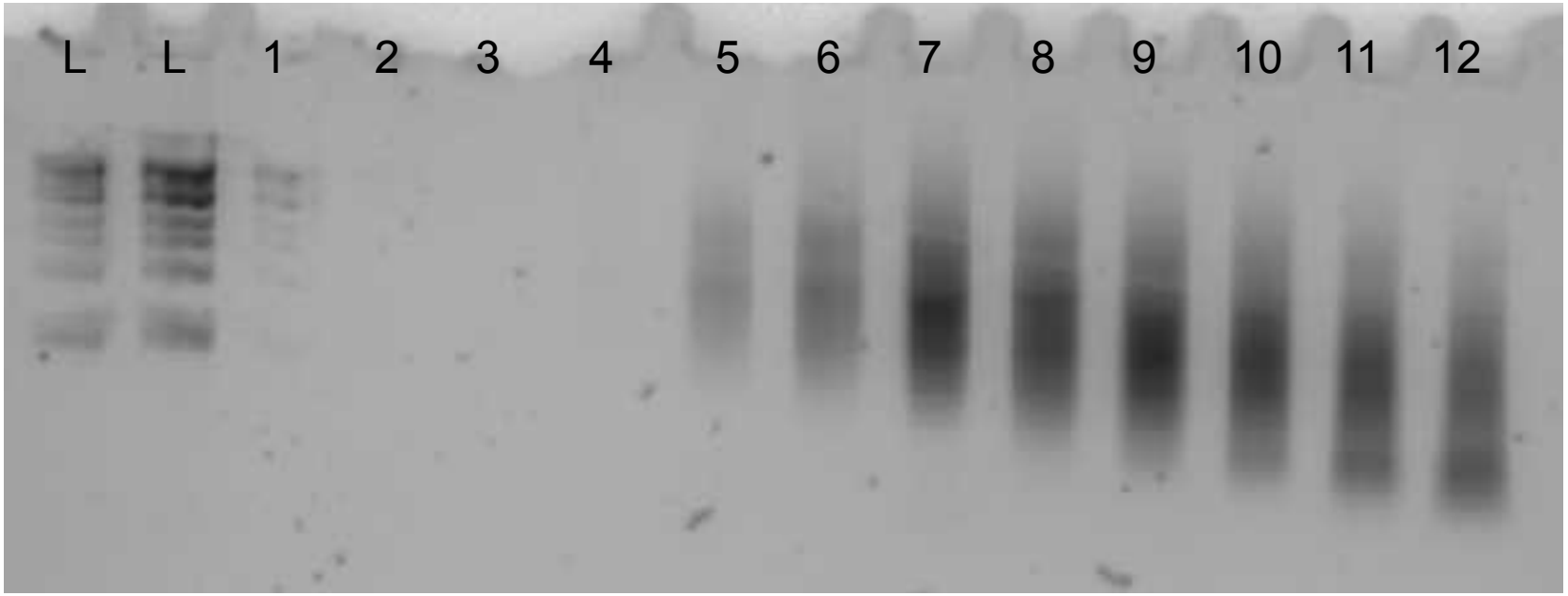


# PCR based cDNA Synthesis

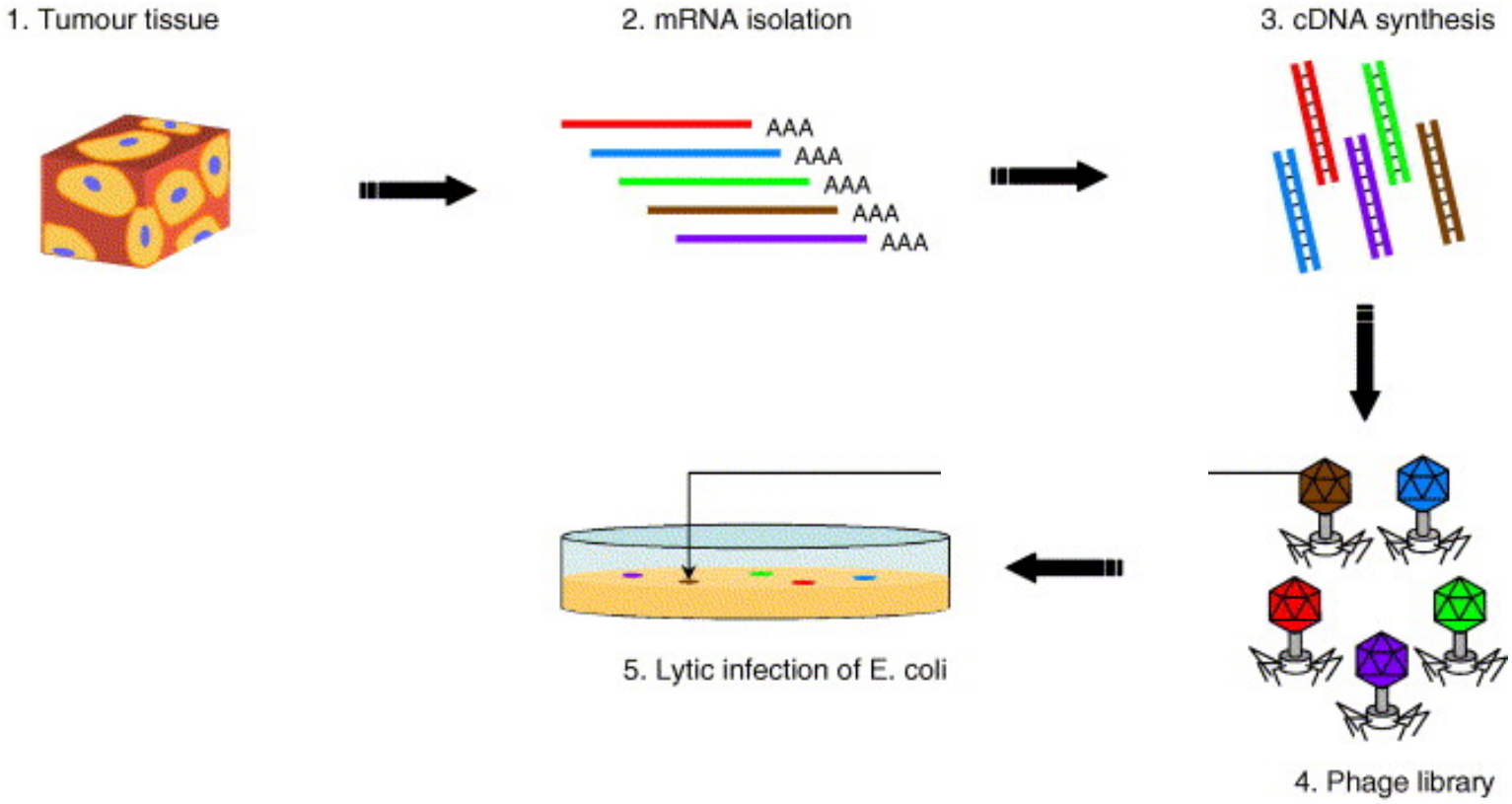
- Faster synthesis protocol
- Starting material necessary is 10% of what the previous protocol called for



# Control cDNA Fractions



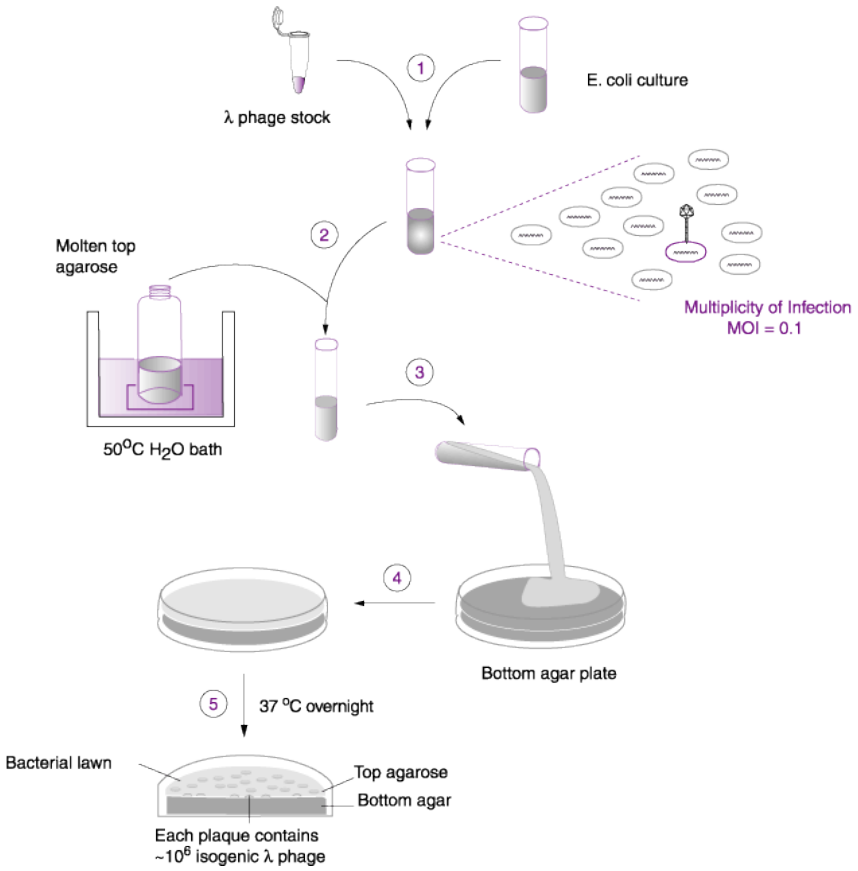
# Modified SEREX





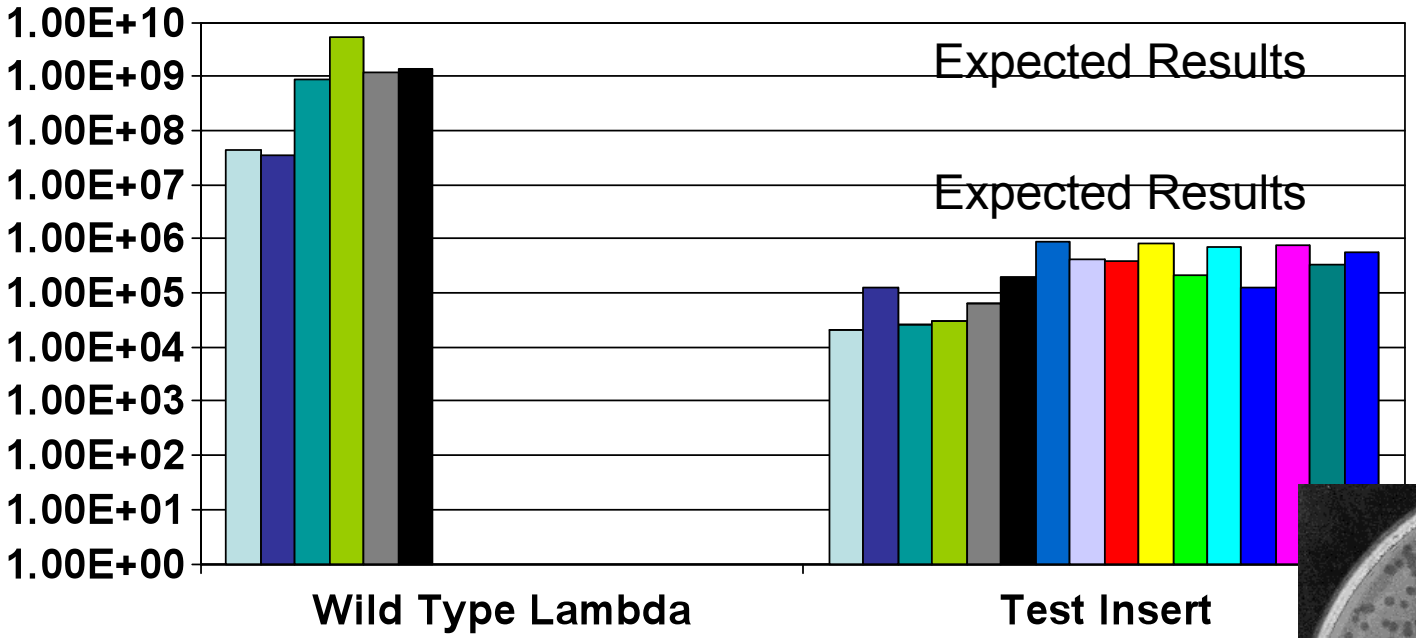

Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

# Control Ligation and Packaging Experiments



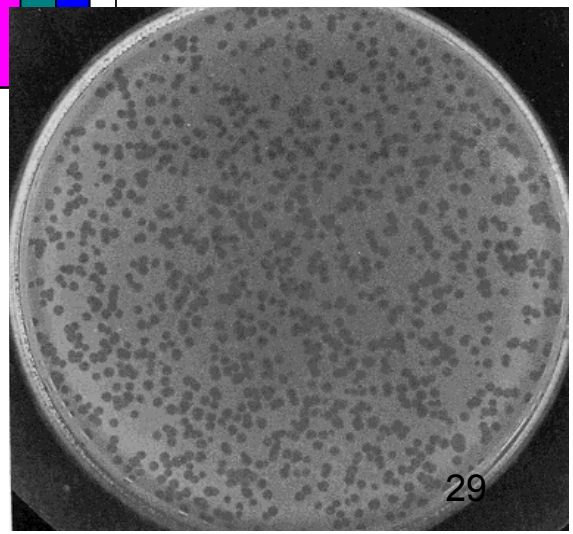

1. Obtained correct titer with wild type phage and packaging extracts
   - Experimental titers < Expected
   - Replaced packaging extracts and obtained experimental titers in the correct range
2. Used test insert to evaluate vector ligation and packaging
   - Experimental titers < expected
   - Suspected bad T4 DNA Ligase

# Control experiments
# Measuring Efficacy of Insertion




WT

Test Insert





Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

# Interim Conclusions

- RNA Isolation
  - High quality extractions were obtained from SkBr3 cells
  - Sufficient quality extractions were obtained from tumor tissue
  - Isolation of RNA lymph node tissue is difficult and better methods must be developed
- cDNA Synthesis
  - cDNA libraries are structurally intact and ready for vector insertion
  - However, variations on synthesis reaction will be explored to increase cDNA yield
- Size Fractionation:
  - Poor product retention yields inconsistent results occur with current methods
  - Faster methods with higher product retention using PCR Clean Up and molecular weight spin columns are currently being developed for this protocol
- Ligation and Packaging Reactions
  - Still in development pending cDNA synthesis reactions

# Project Overview

Aim 1: Generate a phage displayed cDNA expression library representative of tumor antigens

Aim 2: Identify peptides from the library to which patient antibodies bind

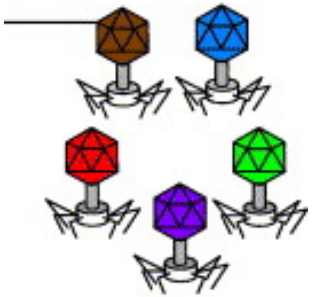
Aim 3: Create scFv libraries from different sources of tissue in the same patient and identify binding scFv antibodies

# Identify peptides from the library to which patient antibodies bind

- What proteins do we expect to identify?
- Why are they important?
- What is the back up plan if we cannot ID them?

# SEREX: Identifying Tumor Associated Antigens

- Bead based assay
  - Collect patient IgG
  - Bind IgG to column
  - Subtract non-specific binders
  - Incubate with phage library of tumor antigens
- Isolate binders and analyze via ELISA



4. Phage library

7. Immunoscreen of antigens using autologous serum



Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

# Project Overview

Aim 1: Generate a phage displayed cDNA expression library representative of tumor antigens

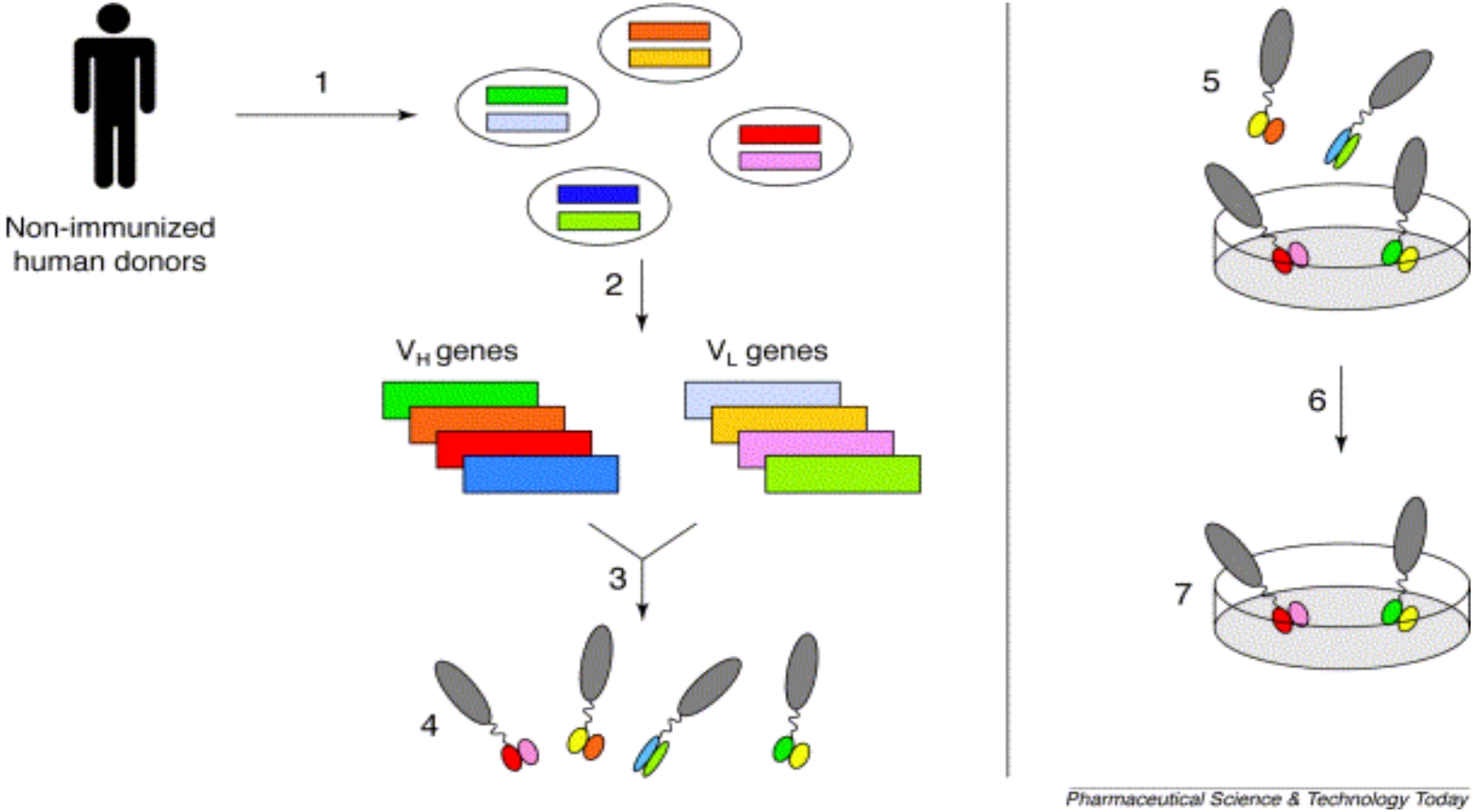
Aim 2: Identify peptides from the library to which patient antibodies bind

Aim 3: Create scFv libraries from different sources of tissue in the same patient and identify binding scFv antibodies

Create scFv libraries from different sources of tissue in the same patient and identifying scFv antibodies that bind

- How do you create an scFv library?
- What kind of autoantibodies do we expect from these libraries and can these antibodies be useful?

# Creation of an scFv library



# Project Overview

- Aim 1: Generate a phage displayed cDNA expression library
- Aim 2: Identify peptides from the library to which patient antibodies bind
- Aim 3: Create scFv libraries from different sources of tissue in the same patient and identify scFv antibodies that bind

Implications for translational science

# Translational Science

At the Bench

- Conjugations to the antibody

At the Bedside

- Knowledge to be gained

# Acknowledgements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Erin Montgomery

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Department of Defense Pre-doctoral Training Award
SD Ireland Breast Cancer Research Fund

# Tumor Autoantibodies: Potential Therapeutic or Irrelevant Phenomenon?

Leah Novinger

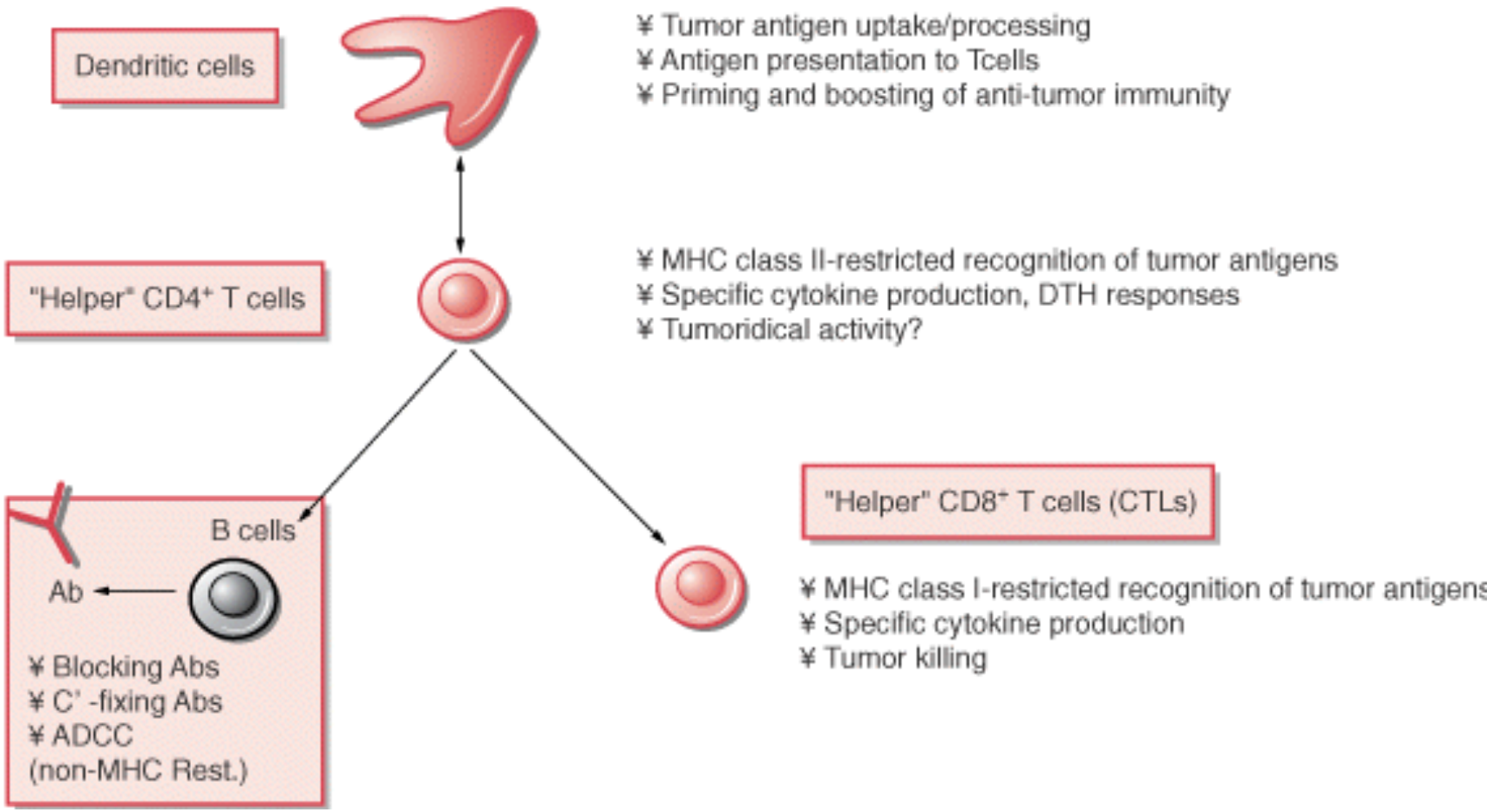
Surgical Research Seminar

May 12, 2010

# Overview - Autoantibodies

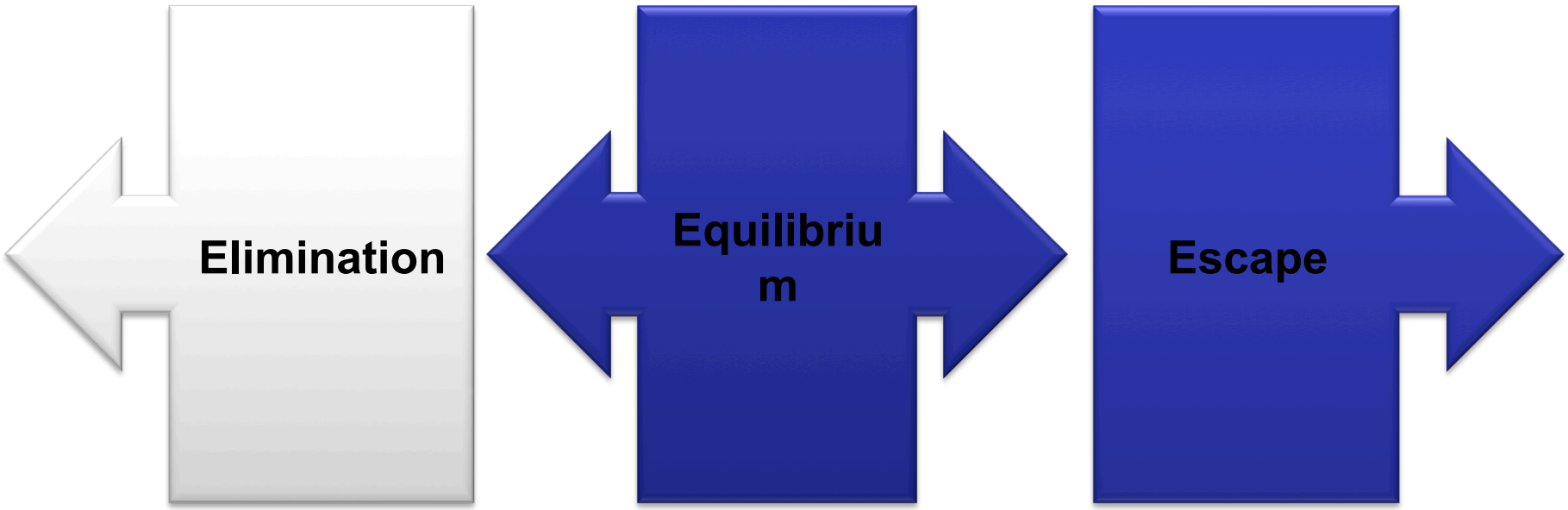
- Overview of Tumor Immunology
- Discovery
  - SEREX
  - SERPA
- Applications
  - Targeted Therapy and Antibodies
  - Cancer Vaccines
  - Adoptive T Cell Therapy
- Conclusions

# TUMOR IMMUNOLOGY




Source: Cancer Medicine, 6th edition

# Tumor Recognition Via Cancer Immunoediting






Source: Dunn GP et al. Nat Immunol. 2002.

# Can the Immune System Destroy Tumors?

- Endogenous IFN-γ protects against tumor formation
- Perforin and RAG$^{-/-}$ lacking mice more susceptible to carcinogens and spontaneous lymphomas




Source: Dunn GP et al. Nat Immunol. 2002.

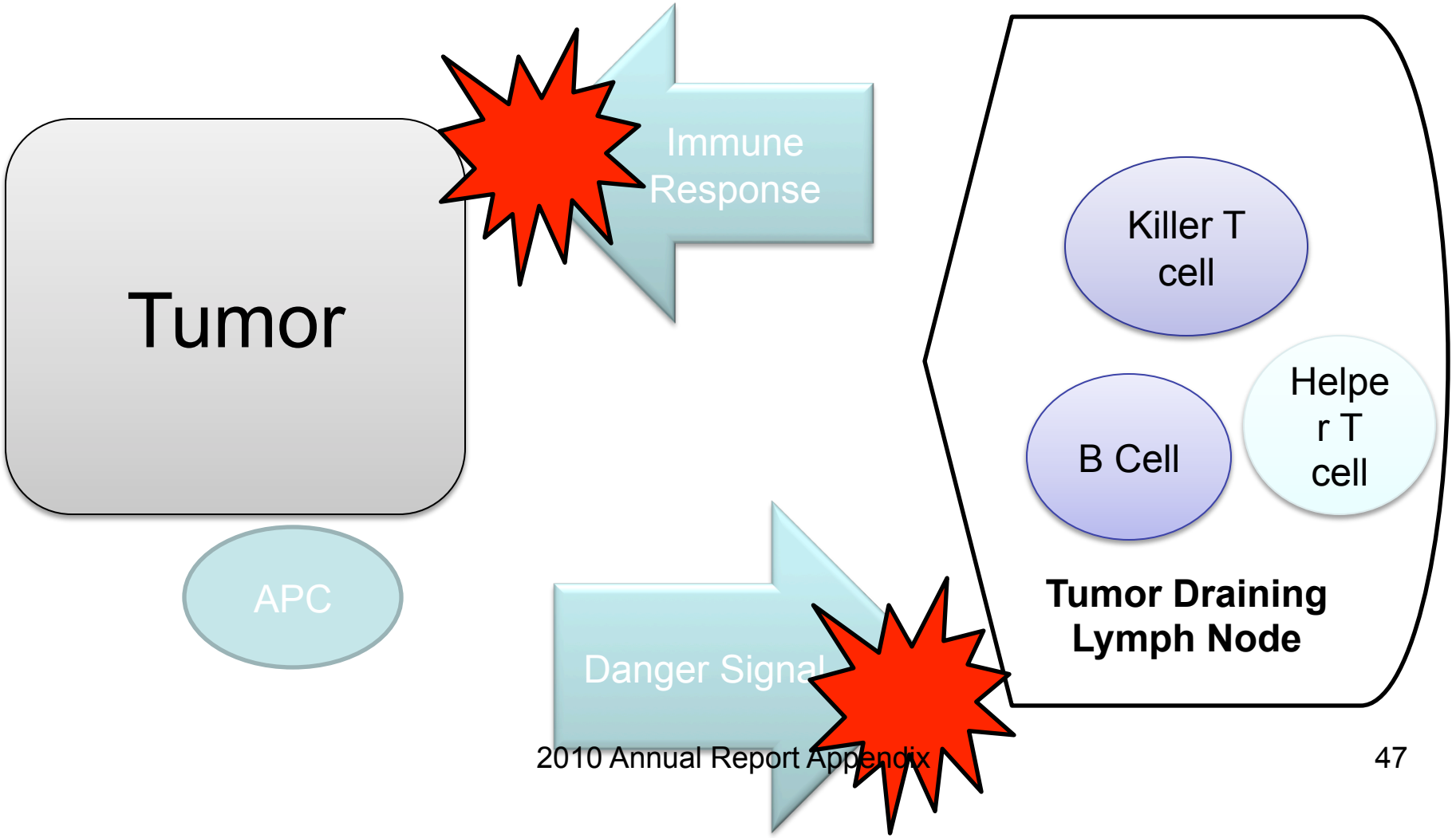
# Can the Immune System Destroy Tumors?

- Patients with primary immunodeficiencies are more like to develop tumors
- Solid organ transplant patients are more likely to develop skin and other solid tumor cancers
- Patients with stem cell transplants are more likely to develop lymphoid neoplasias




Source: Dunn GP et al. Nat Immunol. 2002.

# Why Isn't A Tumor Destroyed By The Immune System?



# Why isn't a tumor destroyed by the immune system?


Tumor Evasion

- MHC Down regulation
- Release of Inhibitory Cytokines

Immune System Dysfunction

- Lack of Danger Signal
- $T_{regulatory}$ Activation
- Tolerance
- Inhibition of Stimulatory Cytokines

# A Positive Correlation Exists Between Tumor Immune Infiltration and Patient Survival

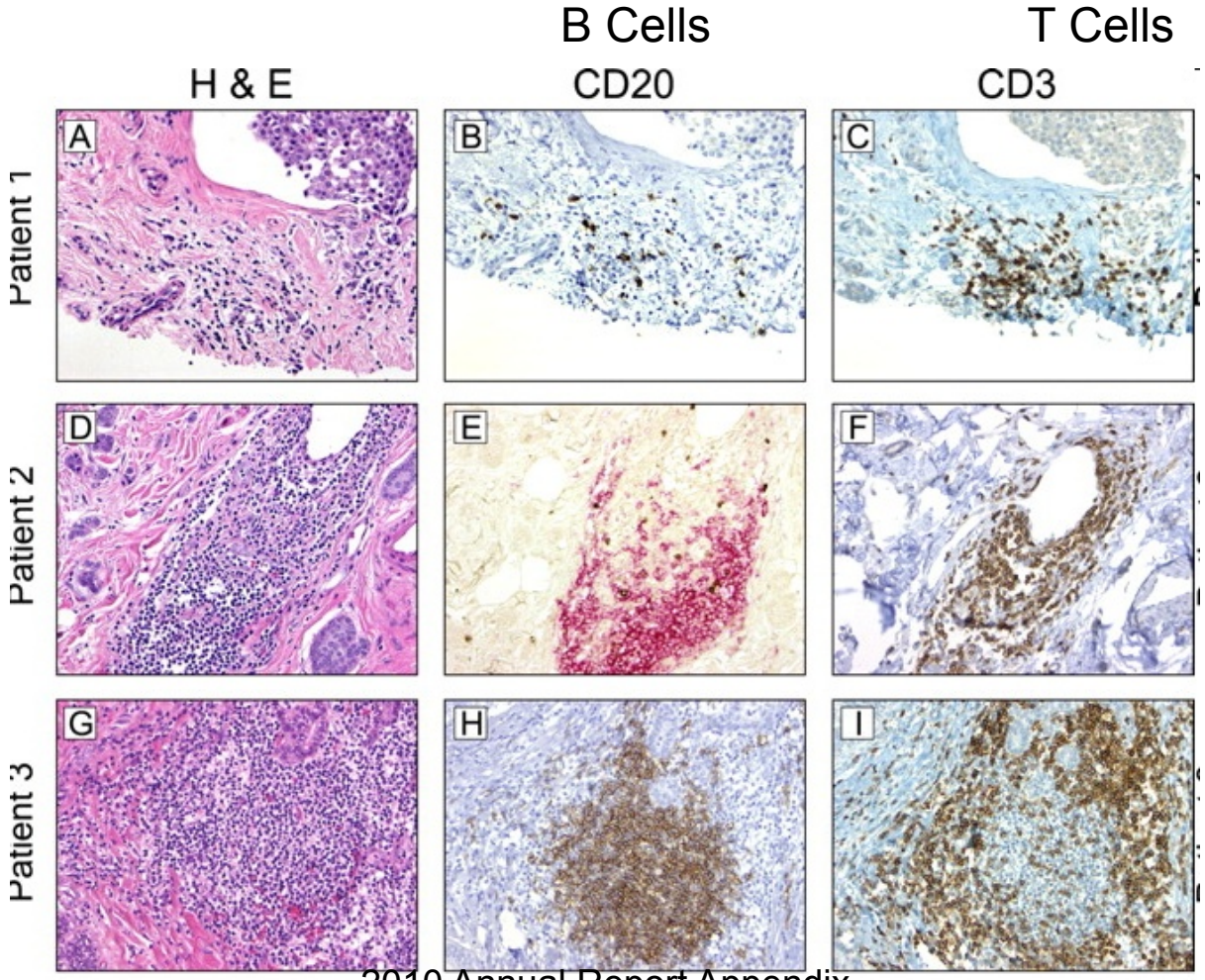
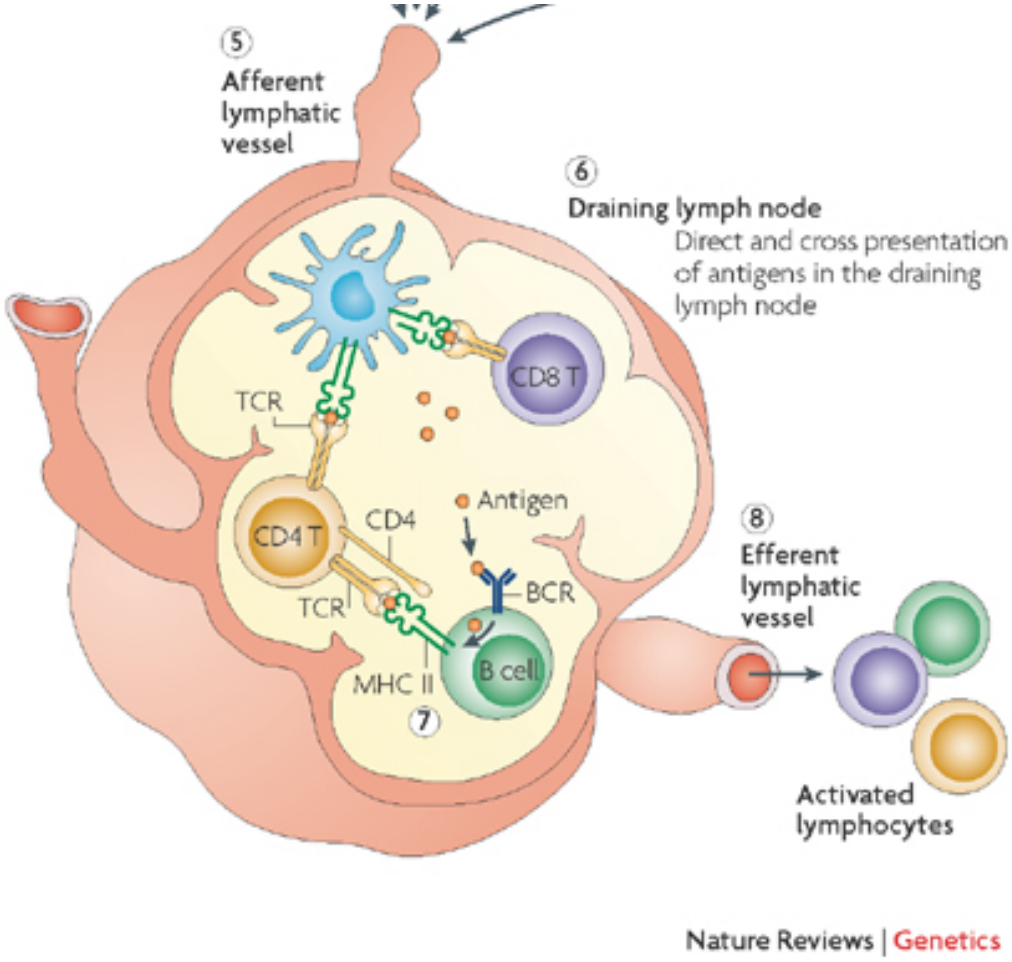





Image source: Coronella et al. J Immunol. 2002 Aug 15;169(4):1829-36.

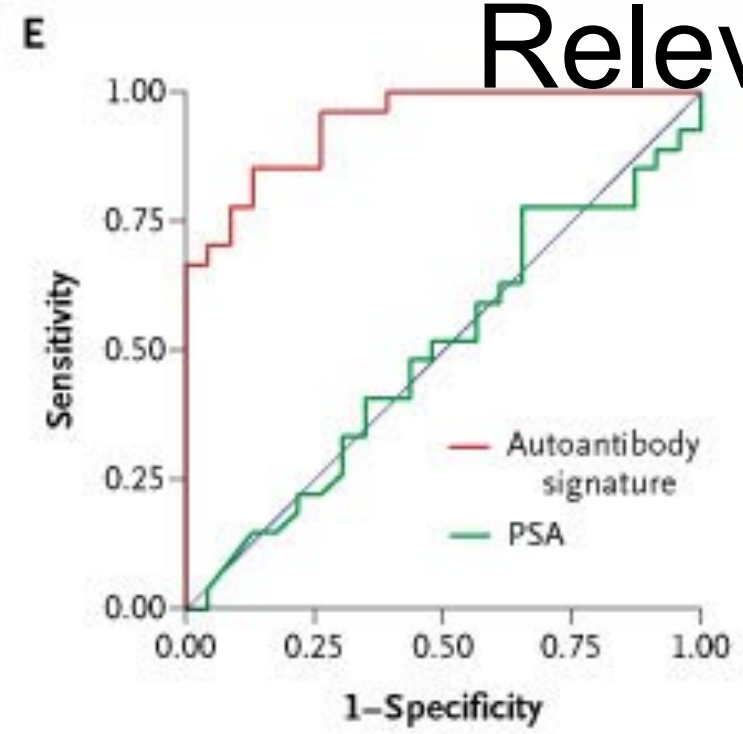
# Autoantibodies: Biological Significance

Effection generation of anti-tumor CTL through cross presentation requires tumor specific antibodies and formation of immune complexes

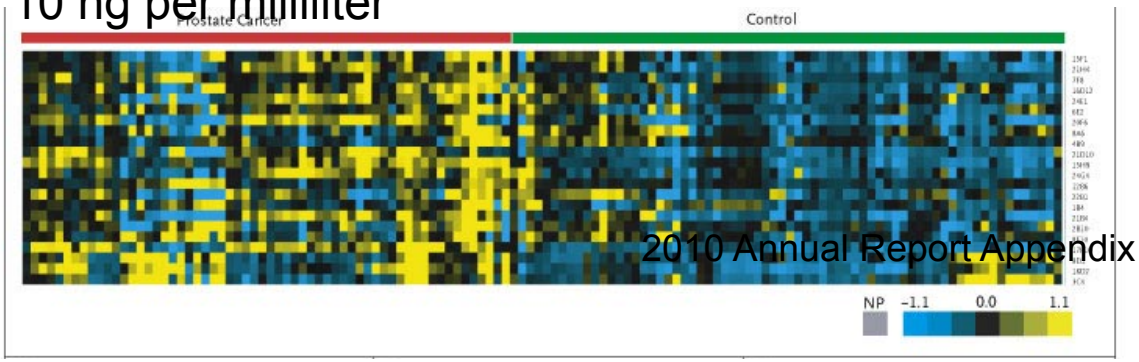



Sources: Kobold, Lutkens et al. 2010, Lee and Jeoung, 2009, Immunobiology, 2001

# Autoantibodies: Clinical Relevance




Performance of the 22-phage-peptide detector in patients with PSA levels between between 2.5 and 10 ng per milliliter

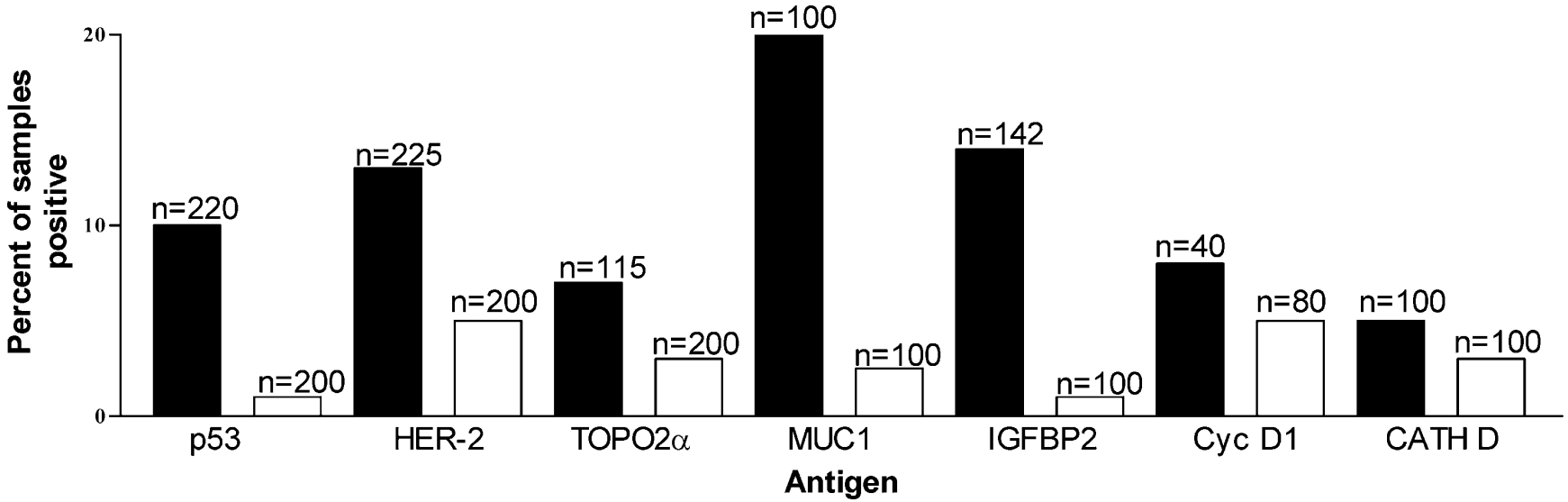



- Phage protein microarrays to analyze serum from patients with prostate cancer
- Created a phage peptide detector of 22 antigens that
- Performed better than PSA at detecting prostate cancer
- 88.2% specific
- 81.6% sensitive

Source: Wang et al. NEJM, 2005

# Autoantibodies: Clinical Relevance

- Detection in high risk patients years before cancer diagnosis
- Antibodies to tumor associated antigens are more frequently detected in sera from breast cancer patients than from normal donors




Shown are the percentages of individuals positive for serum antibody to 7 tumor antigens. Gray columns show the response in patients; white columns show the response in control normal donors. The number of patients or controls tested for each antigen were indicated at the top of the column.

# Tumor Associated Antigens

- Decoy Proteins
- Stem Cell Antigens
- Viral Antigens
- Nucleic Acid Specific Proteins
- Ganglioside like Proteins
- Oncogenic Proteins
- Overexpressed Proteins
- Frameshift Mutations
- Cancer Testis Antigens

# HOW ARE ANTI-TUMOR ANTIBODIES MEASURED?

# Antibody and Tissue Sources

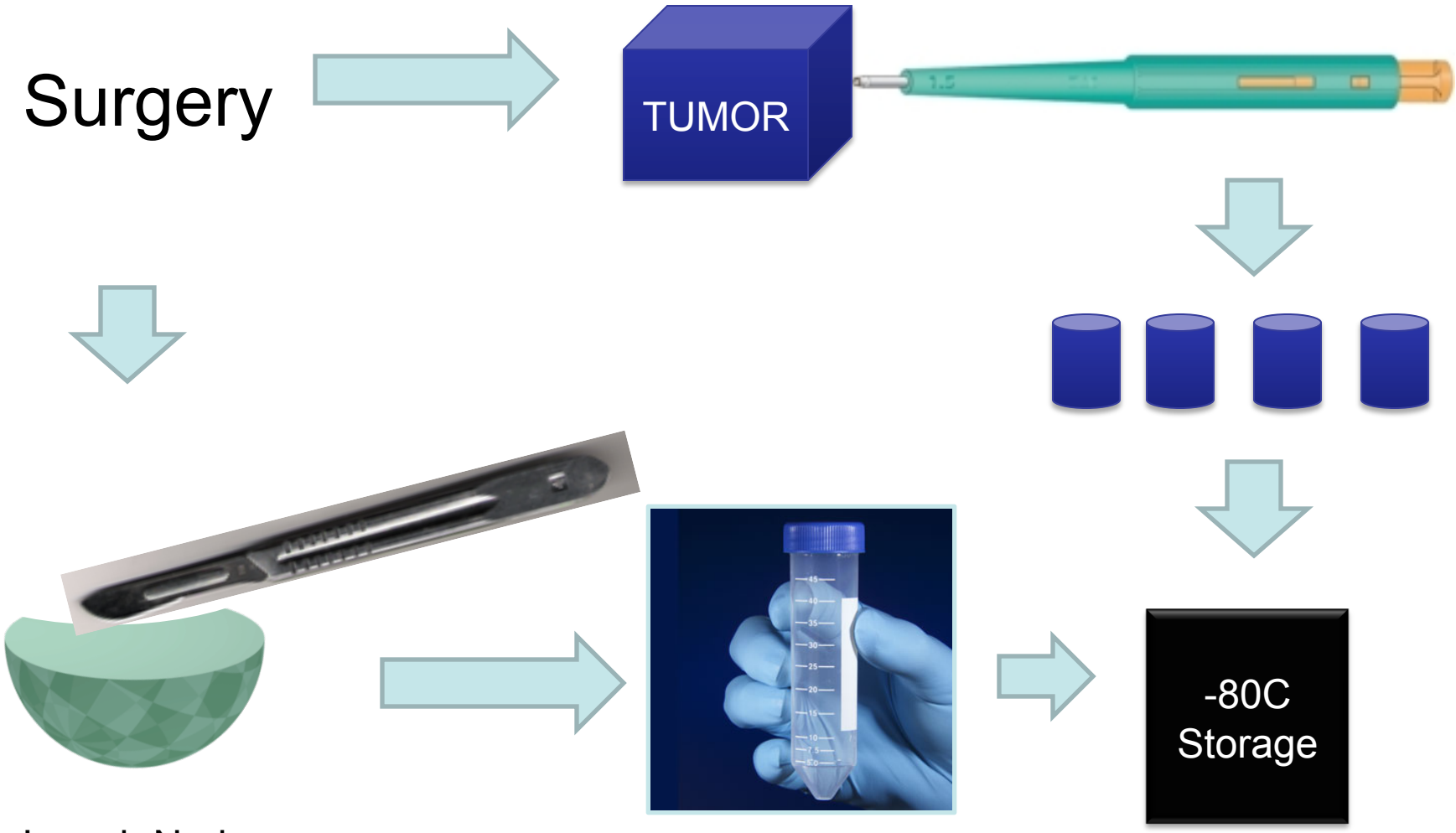
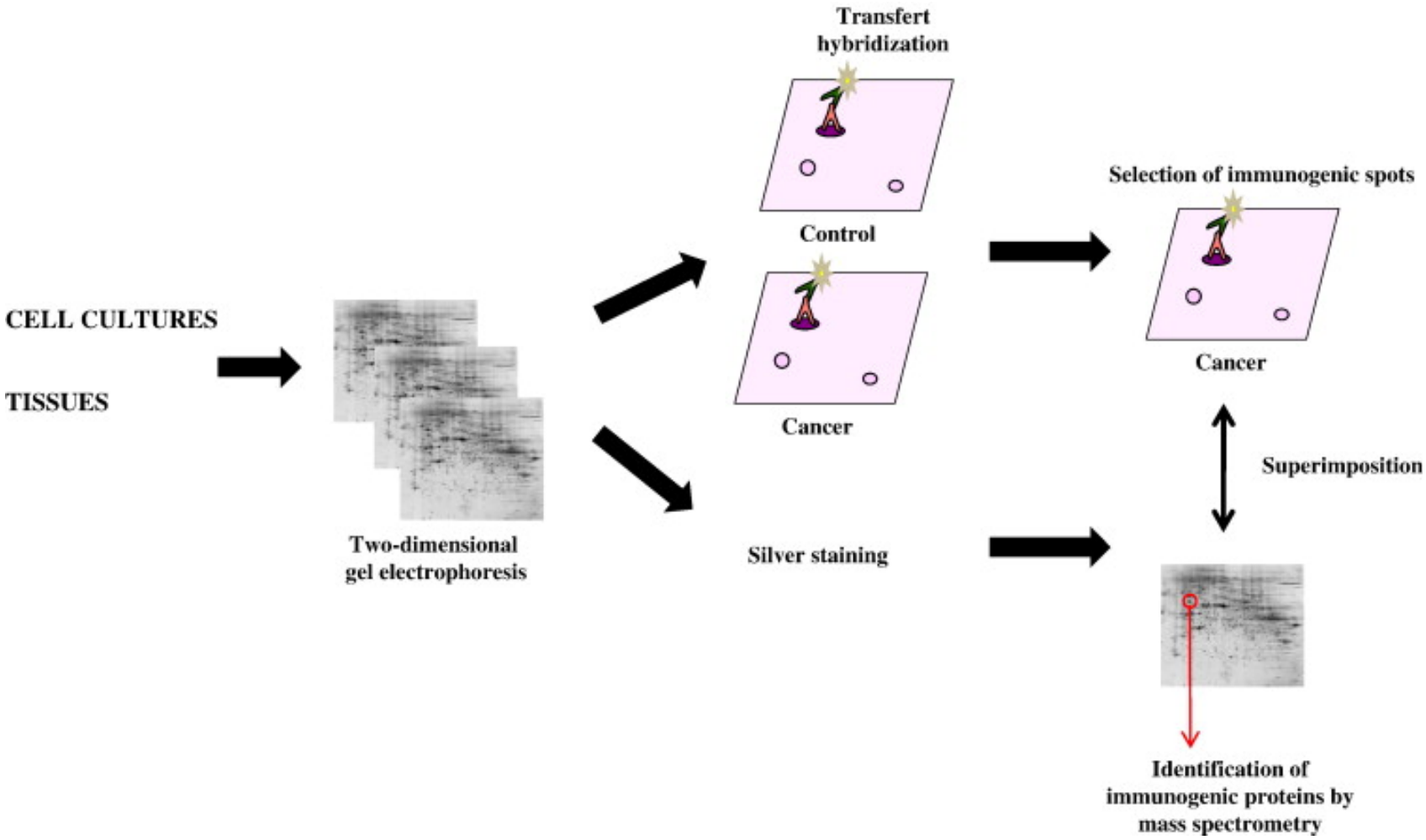





Image Sources:
http://www.sellesmedical.co.uk/store/product/1763-KAI-Disposable-1-5mm-Biopsy-Punch-with-Plunger-Box-of-20-

# Serological Proteome Analysis (SERPA)



# Serological Proteome Analysis (SERPA)

Major Advantage: Can identify antibodies to post-translationally modified peptides and antibodies against multi-protein complexes

Major Disadvantages: Abundant protein necessary to identify signal, Reproducibility

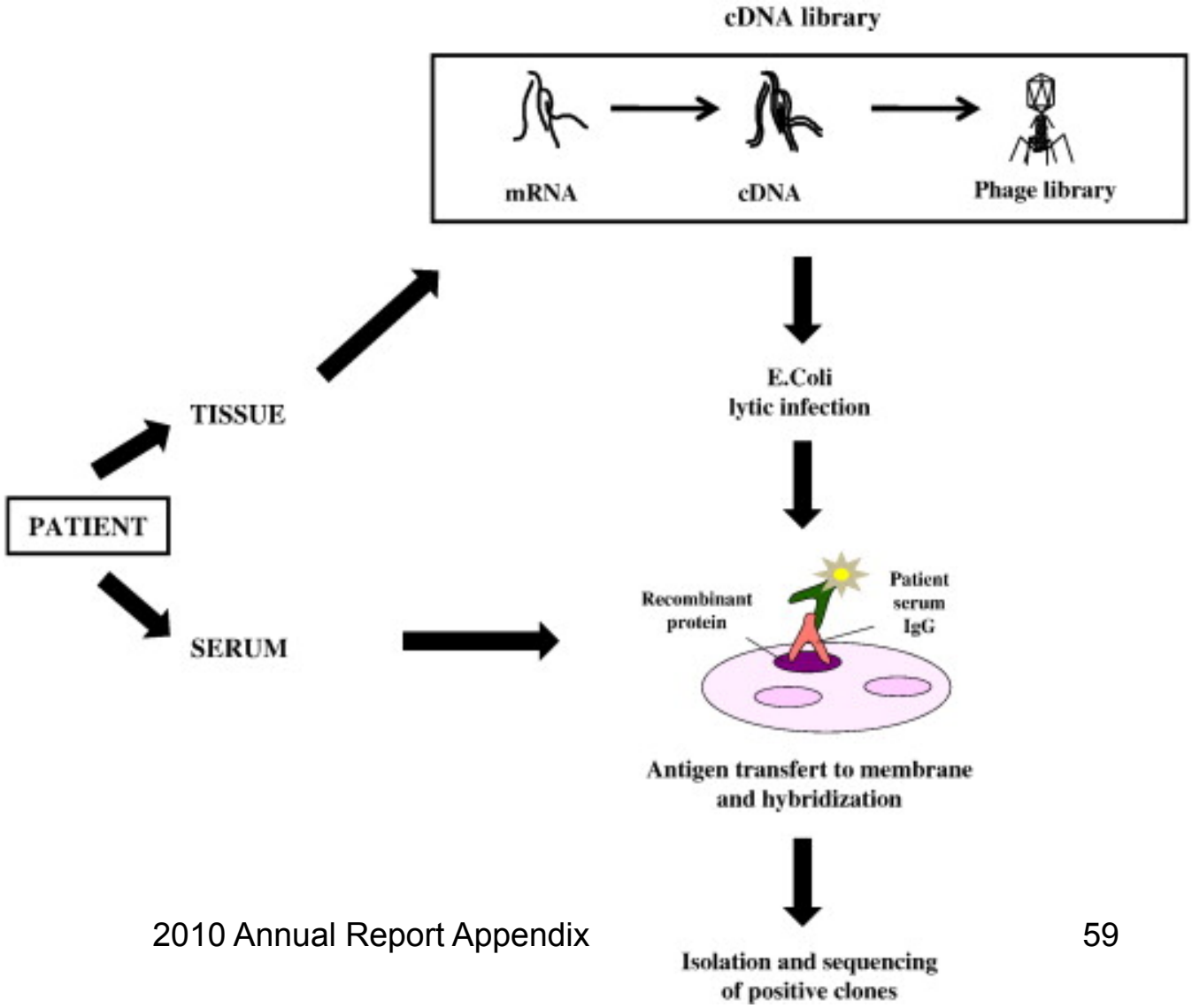


Source: Fernandex Madrid, Cancer Letters, 2005

# Random Peptide Libraries and Molecular Mimicry

- Looks for binding of random peptides, usually 6-10aa long to patient serum after extensive subtraction steps
- Identifies epitopes or mimitopes NOT necessarily original proteins

# Serological Analysis of Tumor Antigens by Recombinant cDNA Expression Cloning (SEREX)



# Serological Analysis of Tumor Antigens by Recombinant cDNA Expression Cloning (SEREX)

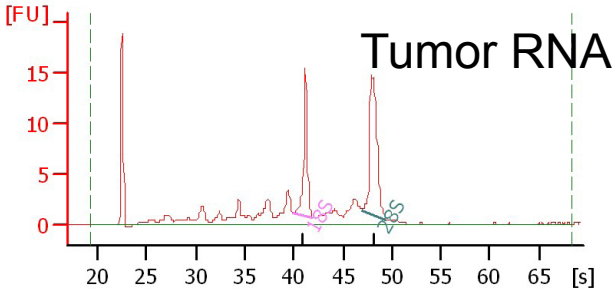
Can identify antibodies to

- Out of frame translation products
- Mutated genes
- New gene products

Cannot identify

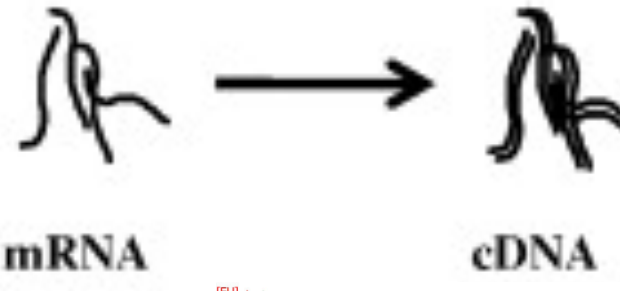
- Antibodies to post-translationally modified peptides
- Conformational epitopes (well)

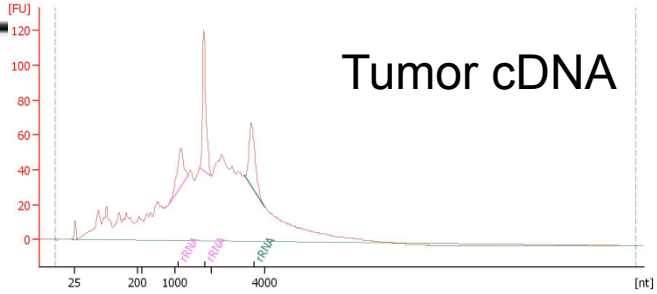
Sources: Desmetz, Cortijo et al. 2009; Kobold, Lutkens et al. 2010; Fernandez Madrid, 2005




Tumor RNA

cDNA library




Tumor cDNA

Development of a protocol for rapid synthesis of cDNA libraries that accurately represent tumor transcripts

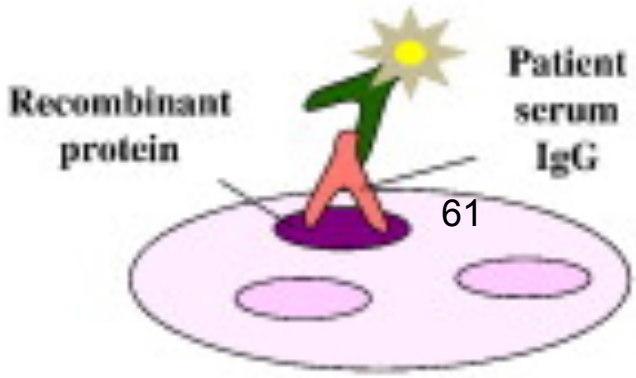
E.Coli
lytic infection

90-95% Vector Ligation
Titers in $10^4$-$10^6$ range

TISSUE

PATIENT

SERUM

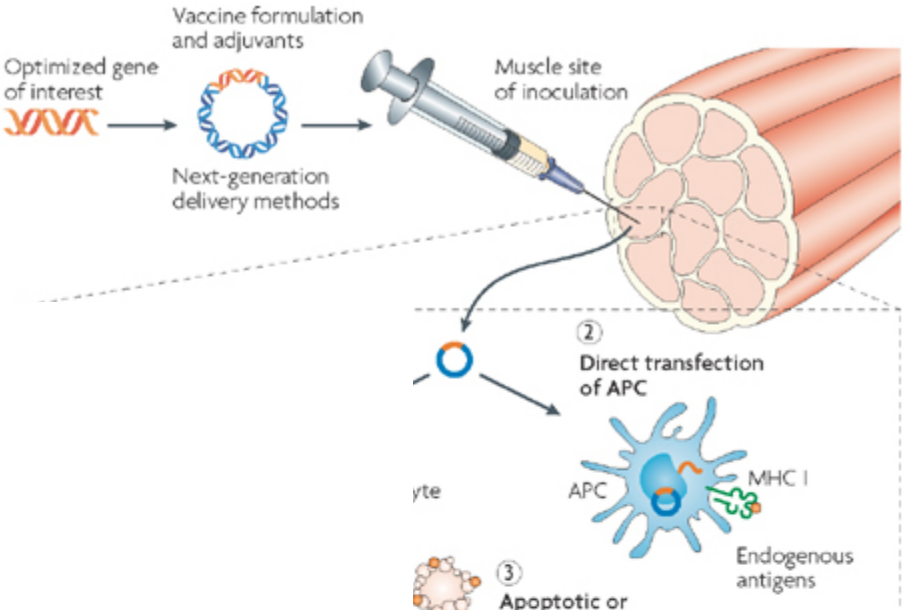


# Interim Conclusions: Identifying Autoantibodies

- SERPA, Random Peptide Libraries, and SEREX are all used to identify autoantibodies and sometimes their corresponding tumor associated antigens
- The Krag laboratory is developing methods to identify autoantibodies through rapid creation of cDNA libraries in phage display

# APPLICATIONS OF AUTOANTIBODIES

# Cancer Vaccines



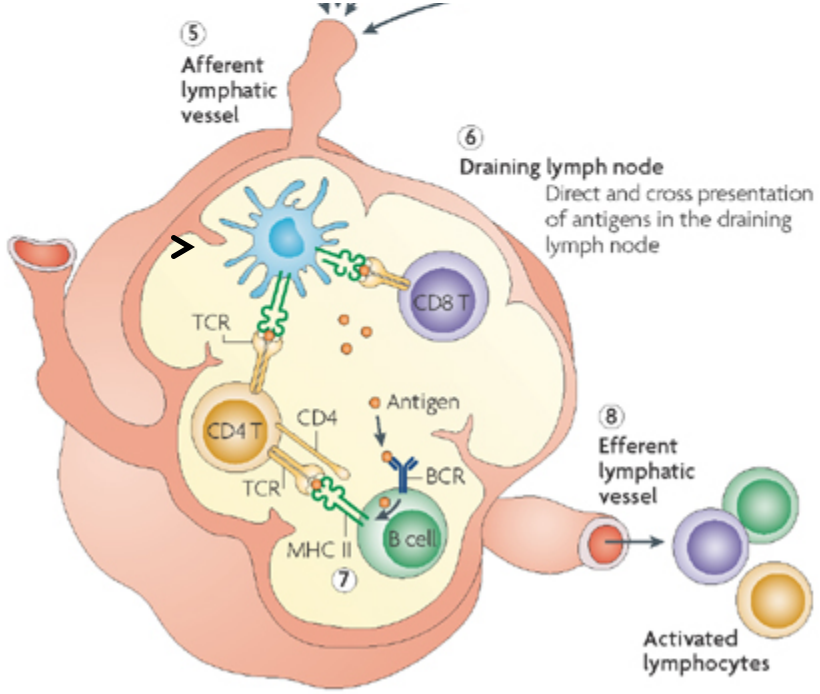
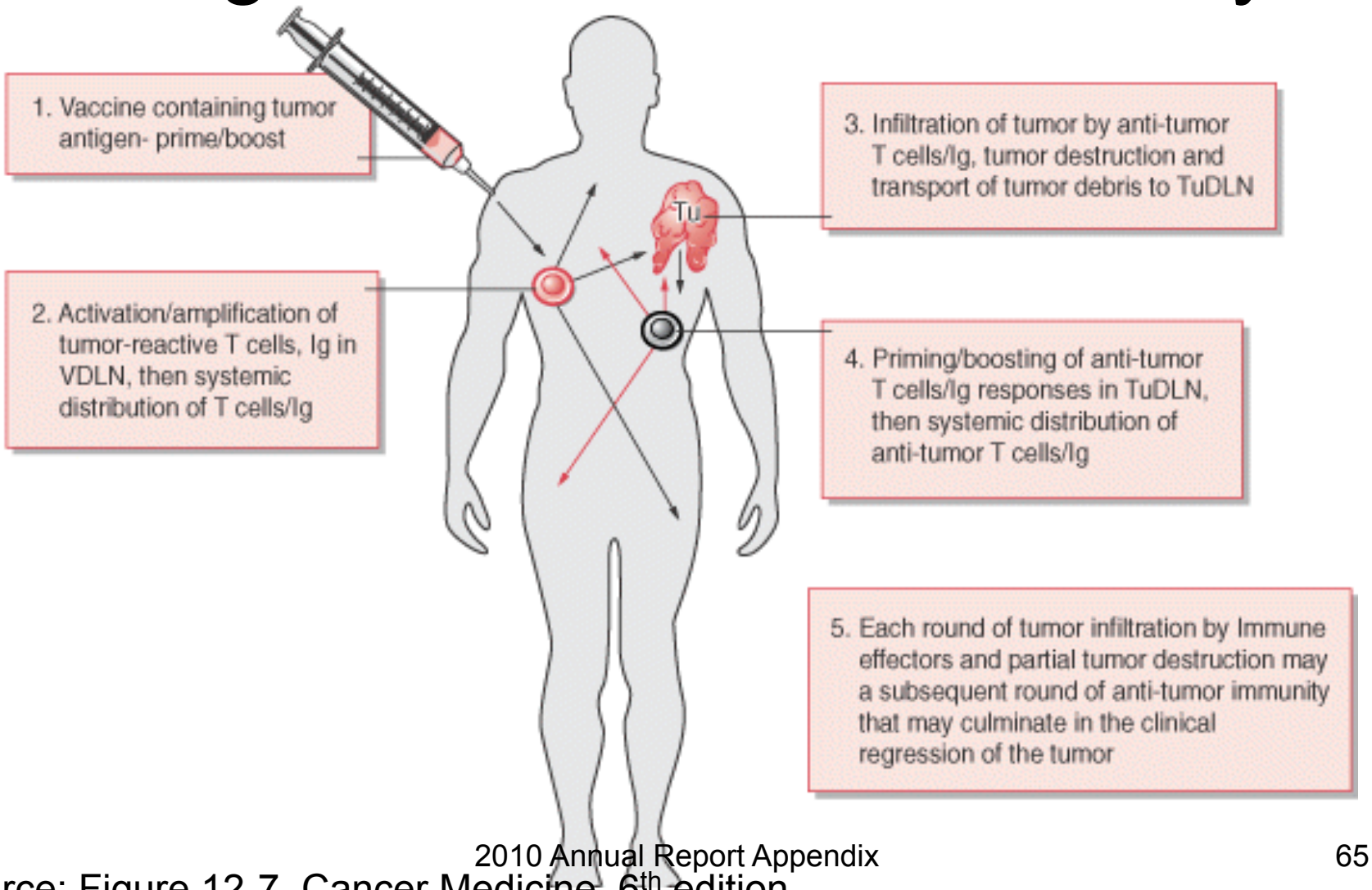



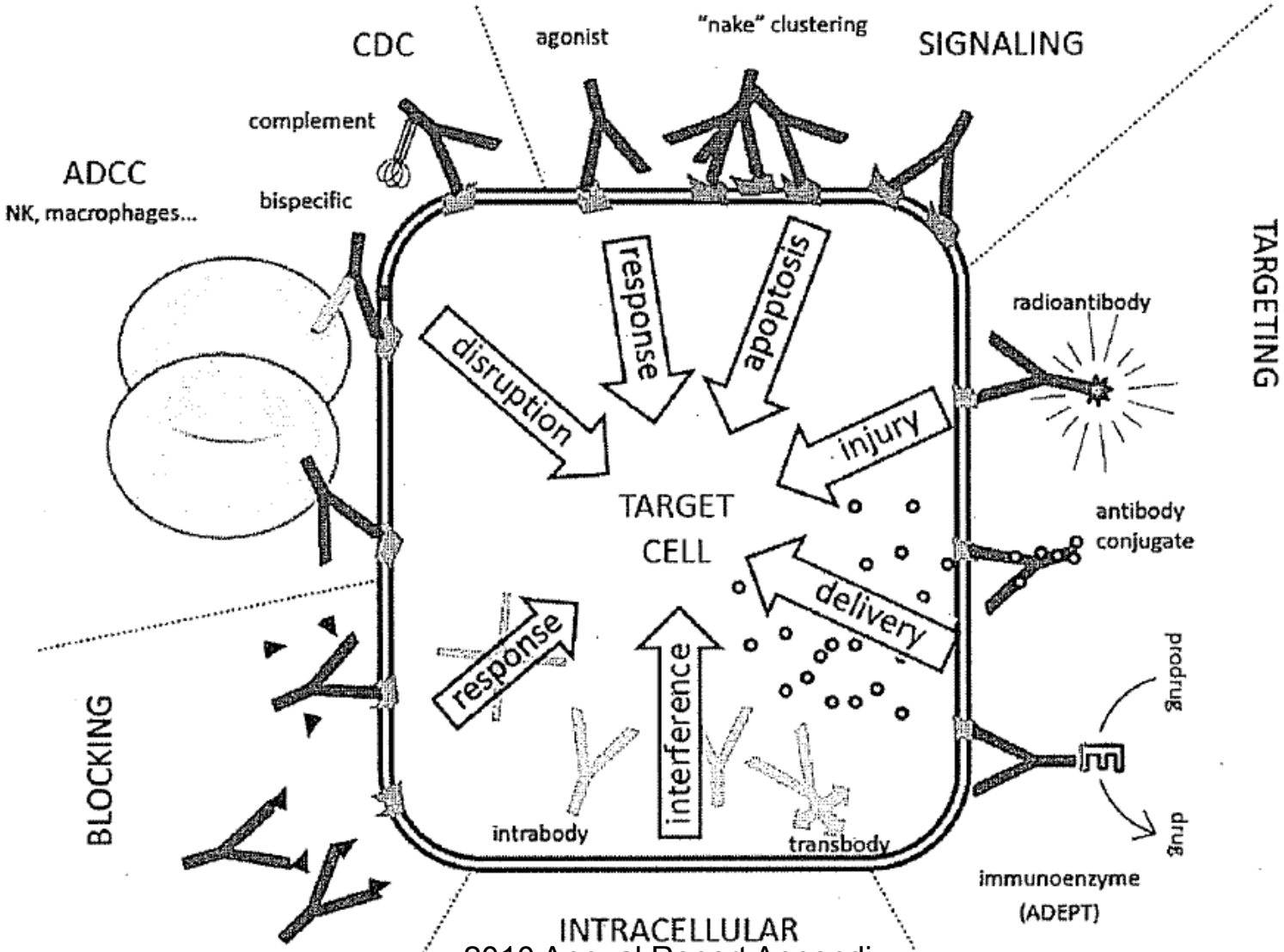

Image Source: Nature Reviews Genetics 9, 776-788 (October 2008)

# Hypothetical model for tumor antigen-based vaccine efficacy



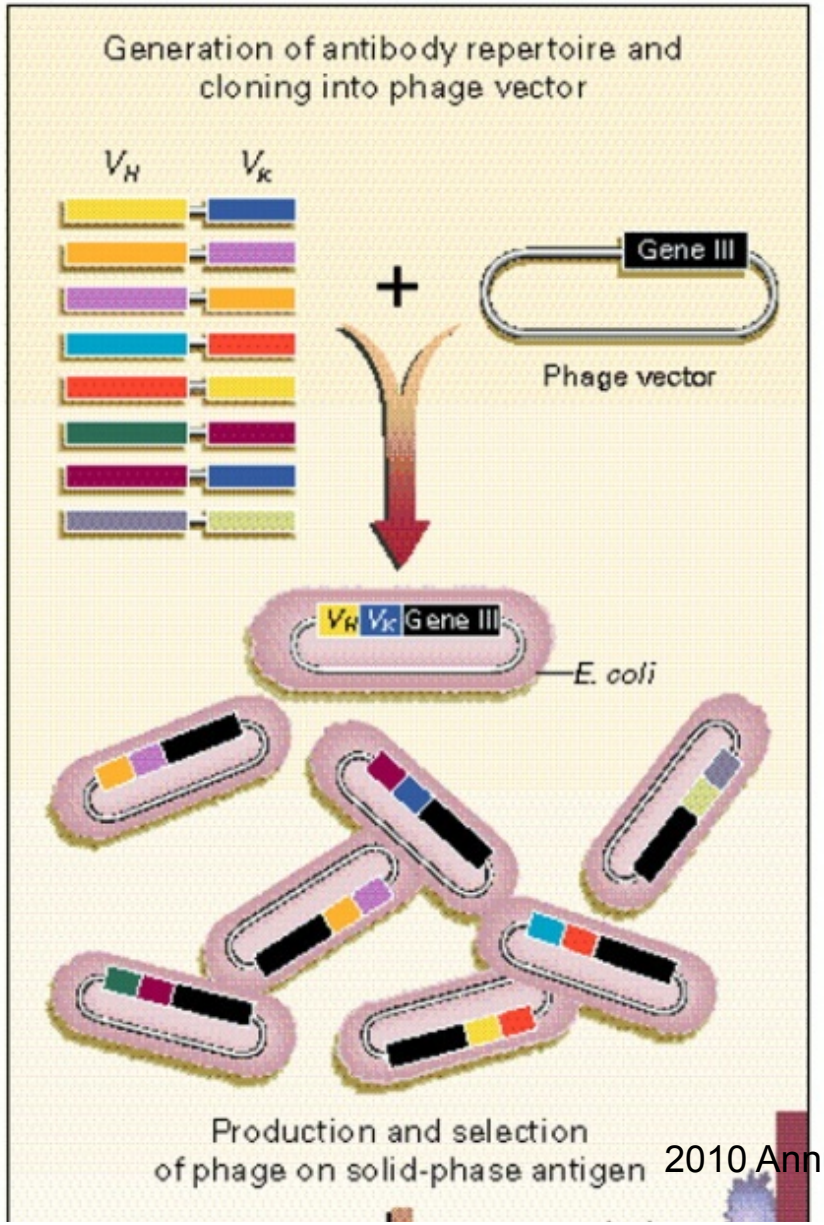

Source: Figure 12-7, Cancer Medicine, 6th edition

# Antibody Therapies




Source: Nieri P Donadio E Rossi S Adinolfi B Podestà A . Curr Med Chem 2009 ;16(6):753-79

# Strategy for Antibody Production with Phage Libraries

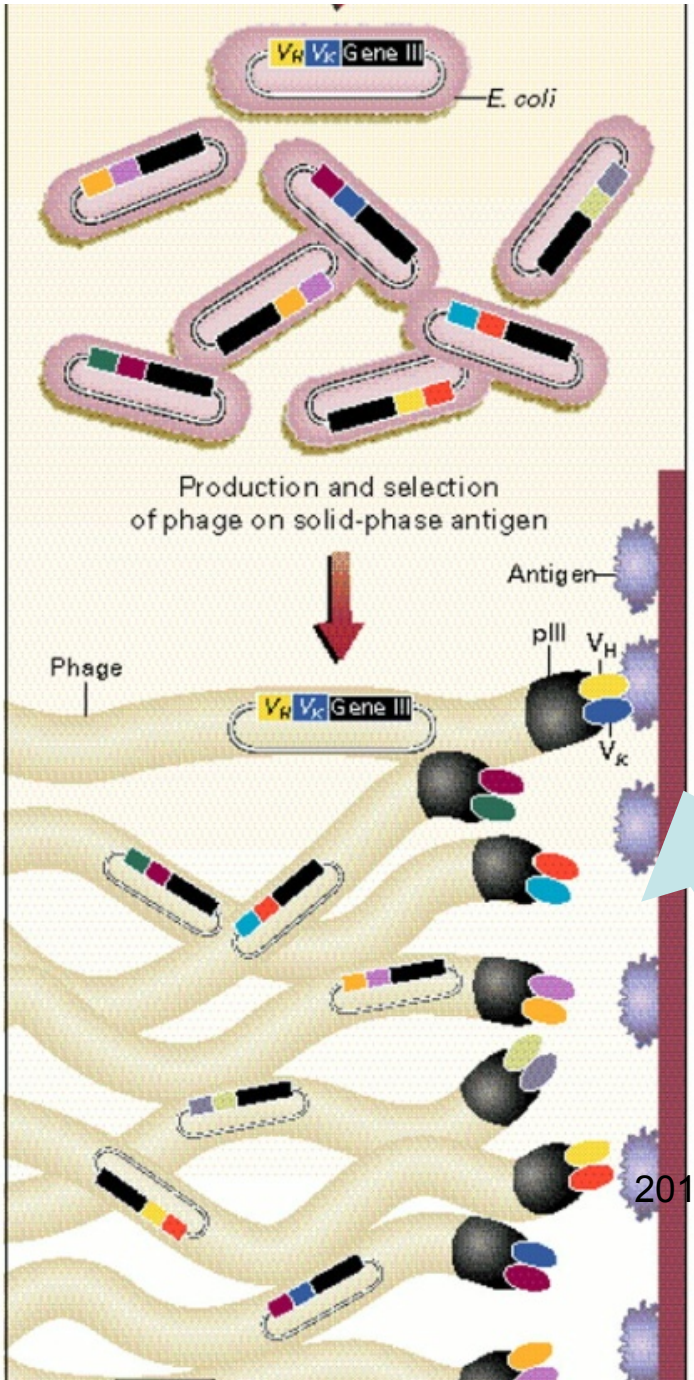


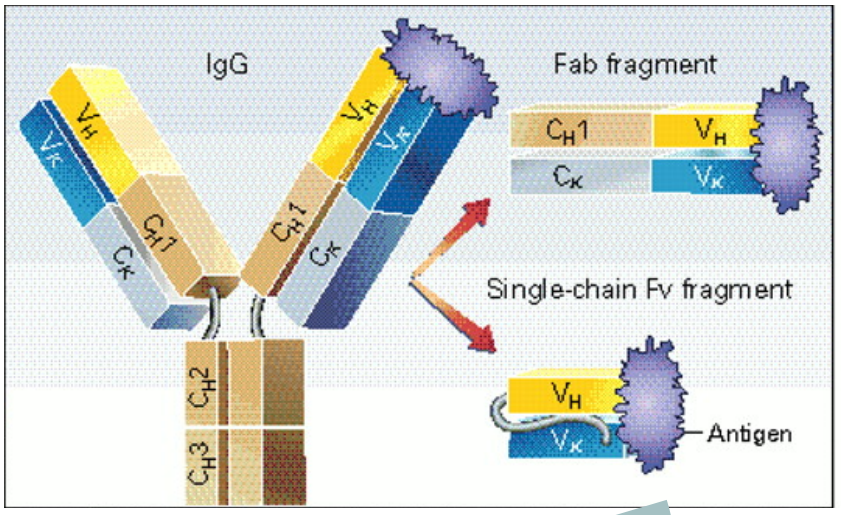

Marks C and Marks J. N E*ngl* J Med 1996;335:730-7



The NEW ENGLAND JOURNAL of MEDICINE

# Strategy for Antibody Production with Phage Libraries




Marks C and Marks J. N Engl J Med 1996;335:730-733



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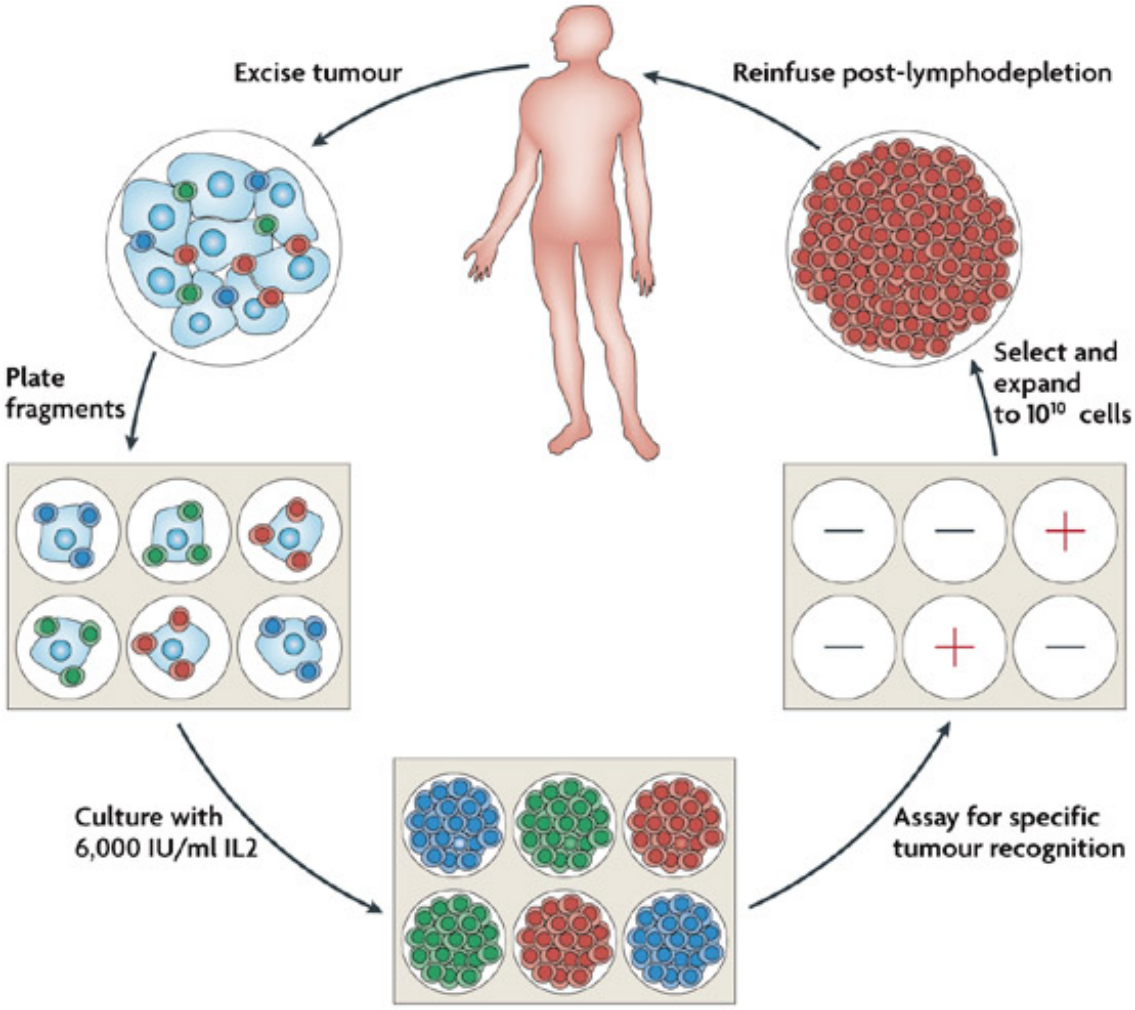
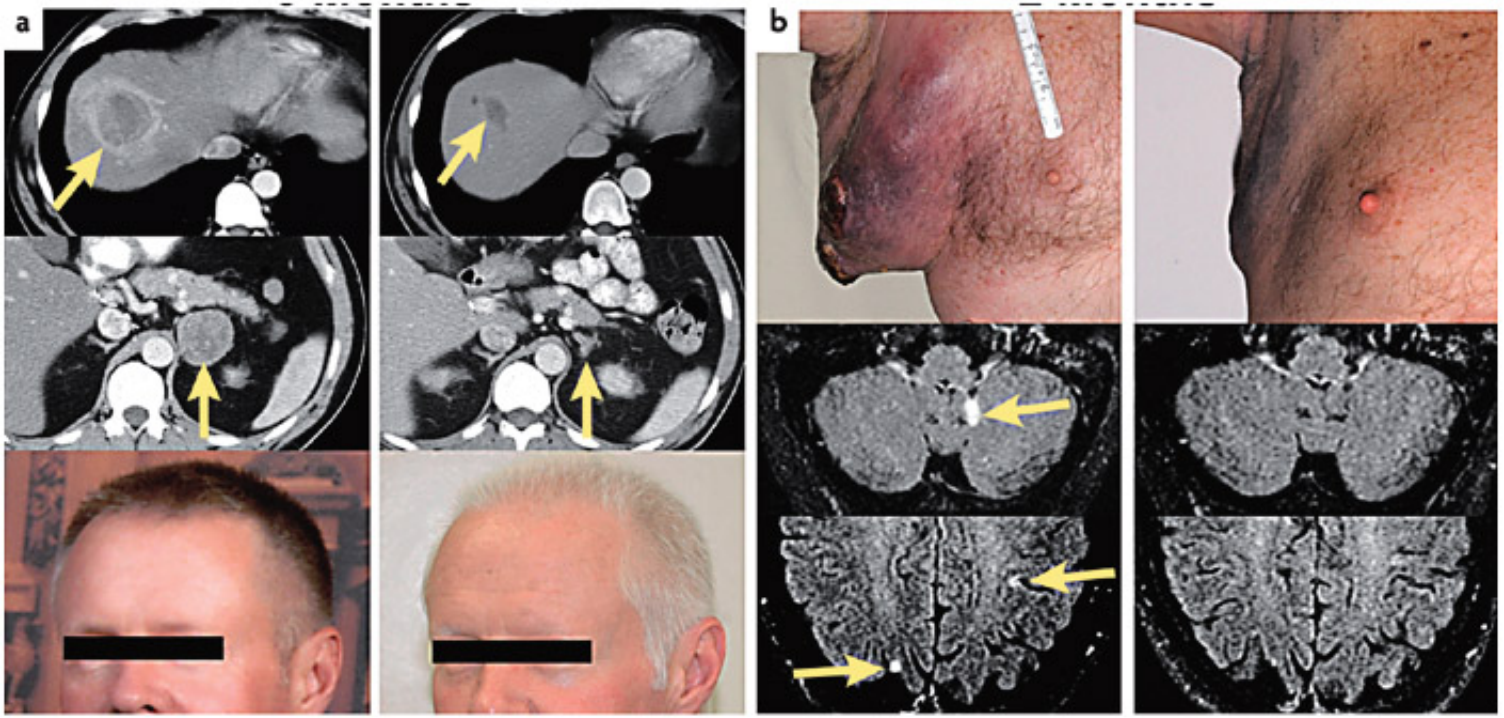
# Adoptive T Cell Therapy



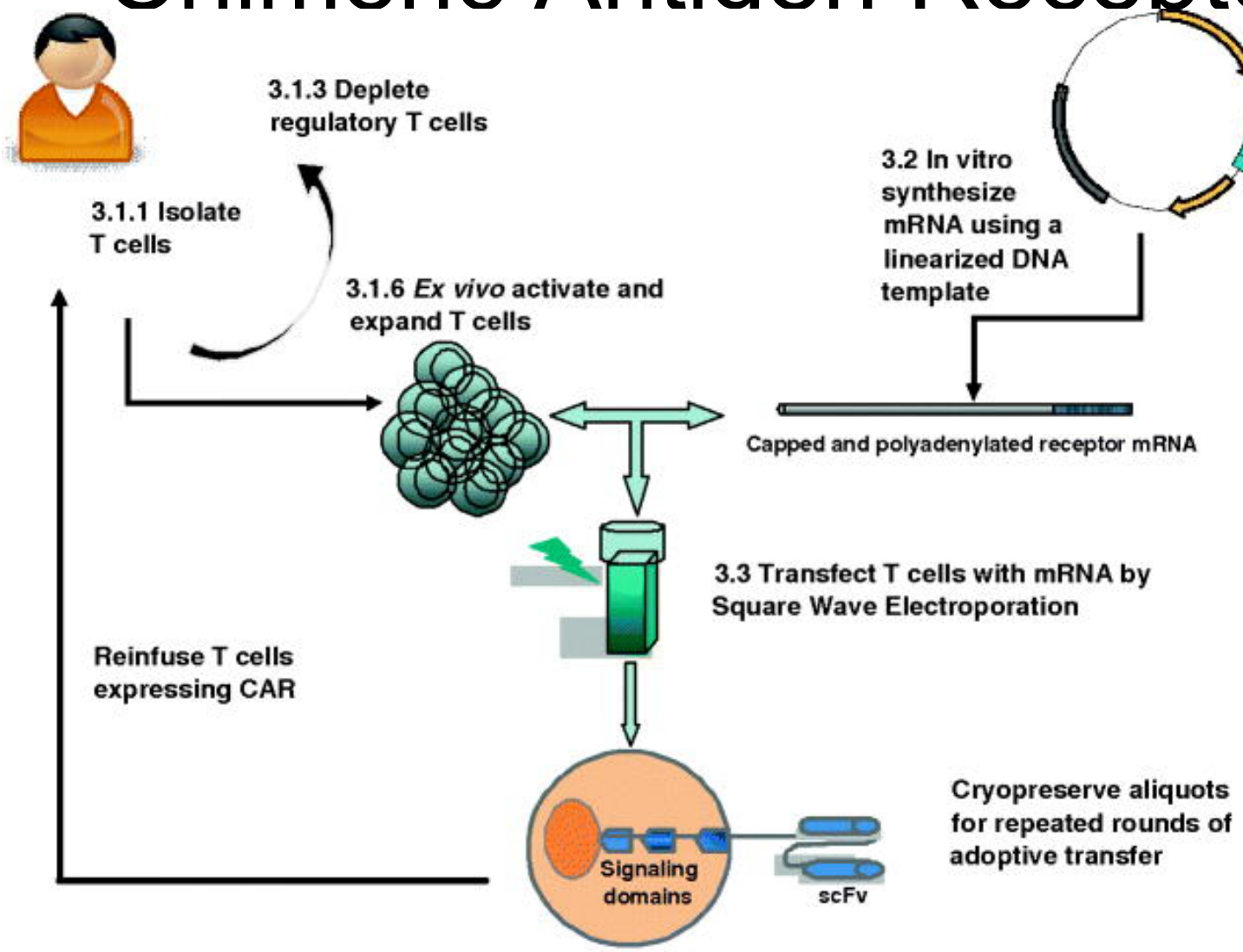

A tumour is excised and multiple individual cultures are established, separately grown and assayed for specific tumour recognition. Cultures with high anti-tumour reactivity are expanded to large numbers (>$10^{10}$ cells) and reinfused into the cancer patient following the administration of a conditioning lymphodepleting chemotherapy. IL2, interleukin 2.

# Adoptive T Cell Therapy




Examples of objective tumour regressions in patients receiving adoptive cell transfer of autologous anti-tumour lymphocytes following a lymphodepleting preparative regimen. **a** | A 45-year-old male with metastatic melanoma to the liver (upper) and right adrenal gland (middle) who was refractory to prior treatment with high dose interferon as well as high-dose interleukin 2 (IL2). He underwent a rapid regression of metastases and developed vitiligo (lower). **b** | A 55-year-old male with rapid tumour growth in the axilla as well as multiple brain metastases from metastatic melanoma that was refractory to prior treatment with high dose IL2 who underwent rapid regression of nodal and brain metastases. Source: Rosenberg, Nat Rev Cancer, 2008

# Chimeric Antigen Receptors




Chimeric antigen receptor T cell

# Interim Conclusions: Applications of Autoantibodies

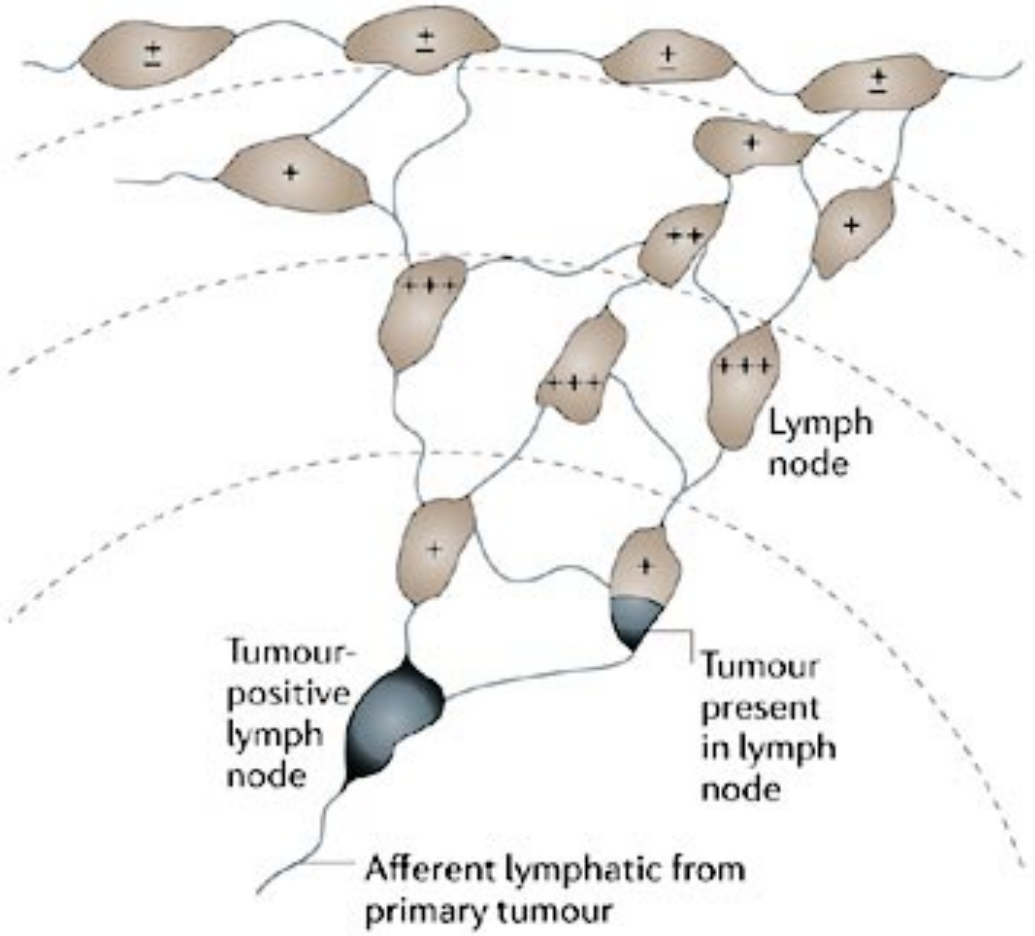
- Identification of tumor associated antigens (TAAs) can lead to effective DNA or Dendritic Cell vaccine and antibody combinations
- A number of different types of antibodies can be developed once an antibody binding to a TAA is identified
- Adoptive T cell therapy utilizes TAAs to specifically target tumors, chimeric antigen receptors utilize antibody binding to activate T cells

# The Role of Sentinel Lymph Nodes in Tumor Immunology

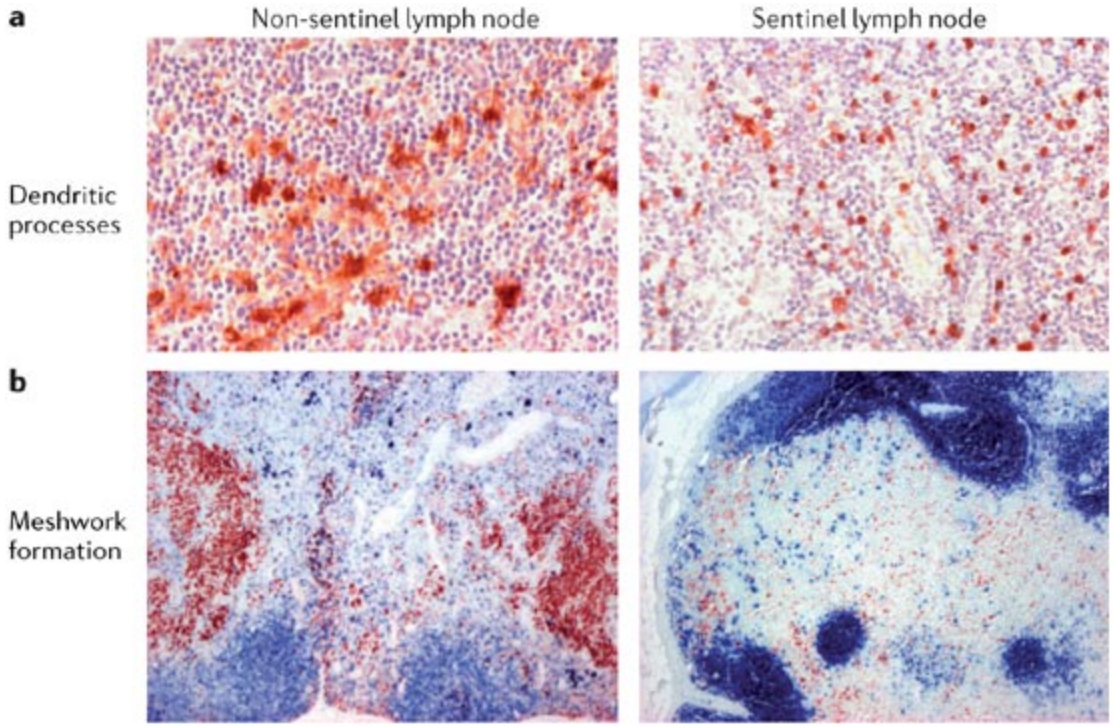
Aassessed by indices including the area occupied by and density of paracortical dendritic cells and T cells, and the frequency of high endothelial venules.

Lymph nodes closest to the tumour receive bioactive tumour products at maximum concentration and are therefore supressed (shown as +).

Source: Cochran et al, Nat Rev Immunology, 2006



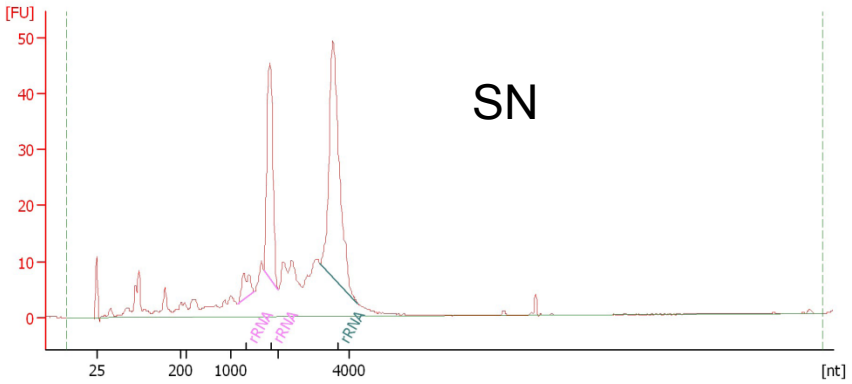
# Immune suppression in sentinel lymph nodes

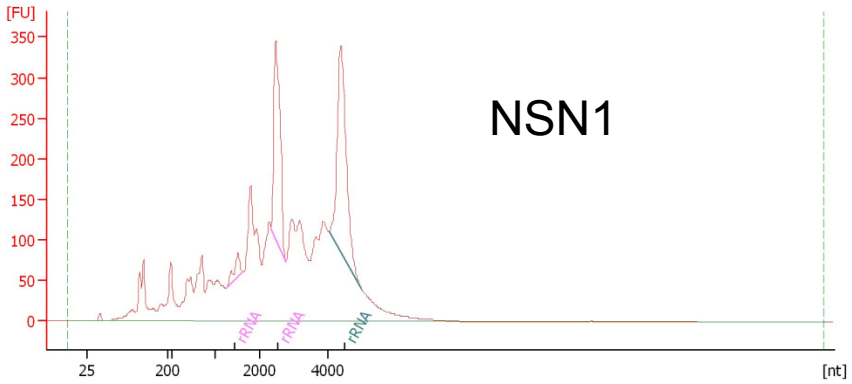


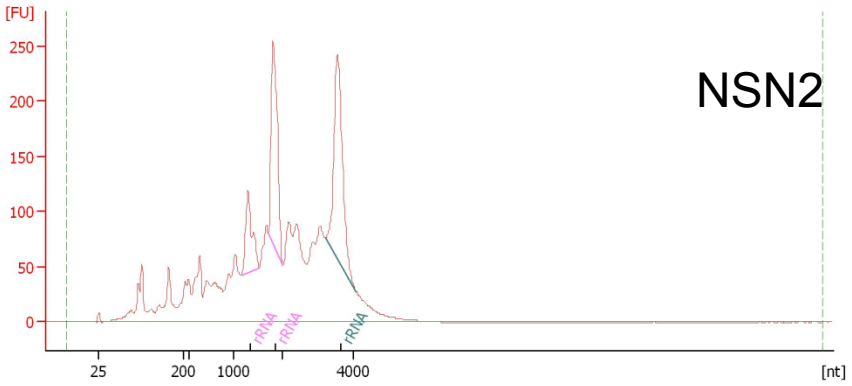


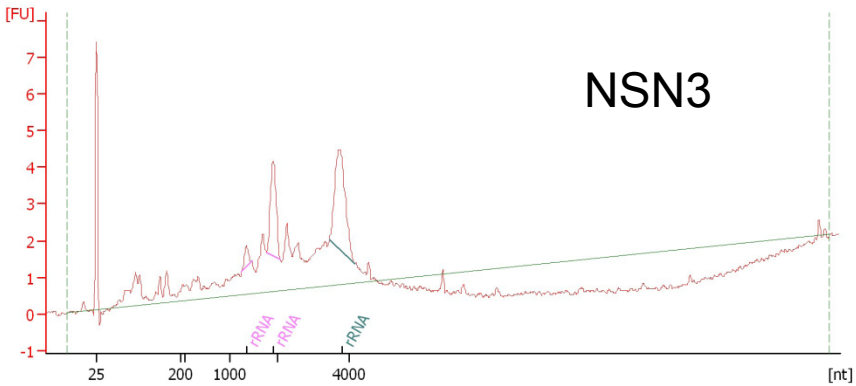

**a** | Abundant polydendritic CD1a$^+$ paracortical dendritic cells (DCs; stained red) are found in a non-sentinel lymph node, whereas there are fewer, poorly dendritic and non-dendritic CD1a$^+$ paracortical DCs in a sentinel lymph node. **b** | Abundant fascin$^+$ paracortical DCs (stained red) form complex meshworks in a non-sentinel lymph node. In a sentinel lymph node, fascin$^+$ paracortical DCs are sparse and show no tendency to form meshworks. Source: Cochran et al, Nat Rev Immunology, 2006

# Lymph Node RNA Analysis



# Conclusion

- Immunoediting: Elimination, Equilibrium, Escape
- Discovery
  - SEREX: flexible cDNA based method allows for different comparisons of high titer IgG
  - SERPA: 2D Electrophoresis captures abundant antigens in their native state
- Applications
  - Targeted Therapy and Antibodies
  - DNA and DC vaccines hold promise, anti-tumor antibodies likely will play a role
  - Adoptive T Cell Therapy has yet to have success in solid tumors other than melanoma, but chimeric antigen receptors have great potential
- Immune response happens in tumor and tumor draining lymph node. Both are sources of B cells, but there is evidence for immune suppression in the SLN.


Questions?

# A high molecular weight melanoma-associated antigen-specific chimeric antigen receptor redirects lymphocytes to target human melanomas.

Burns WR, Zhao Y, Frankel TL, Hinrichs CS, Zheng Z, Xu H, Feldman SA, Ferrone S, Rosenberg SA, Morgan RA.

Cancer Res. 2010 Apr 15;70(8):3027-33.

Presented by: Leah Novinger
MD/PhD Seminar
May 13, 2010

# Overview

- Adoptive T Cell Therapy
- Chimeric Antigen Receptor Therapy
- Measuring Response Ex Vivo and In Vivo
- Approach in Melanoma
- Approach in Other Cancers
  - MUC

# ADOPTIVE T CELL THERAPY

# MD/PhDs Rock!

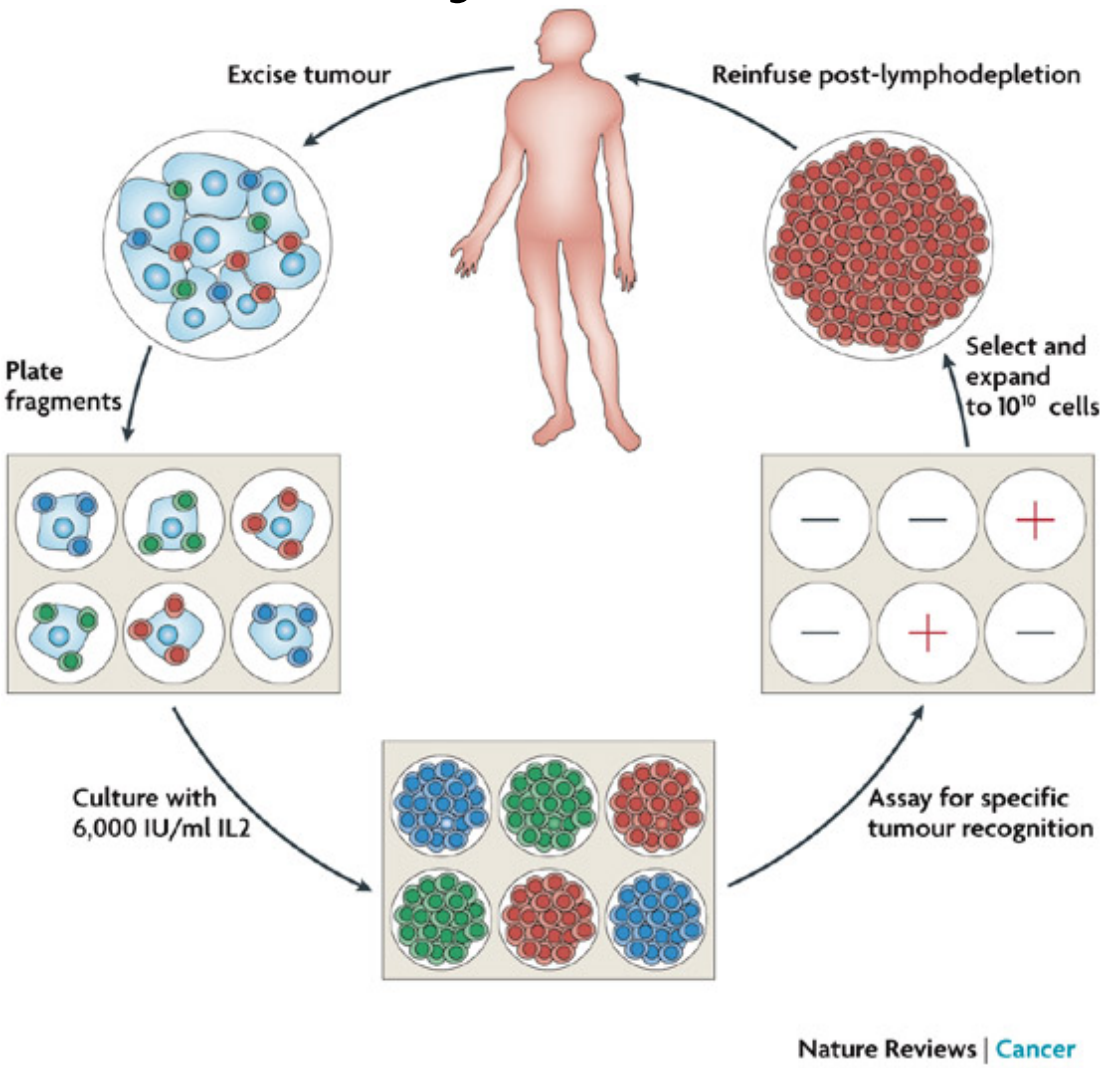
Steven A. Rosenberg

- B.A. The Johns Hopkins University
- M.D. The Johns Hopkins University
- Ph.D. in Biophysics at Harvard University.
- After completing his residency training in surgery in 1974 at the Peter Bent Brigham Hospital in Boston, Massachusetts Dr. Rosenberg became the Chief of Surgery at the National Cancer Institute, a position he has held to the present time.

# Adoptive T Cell Therapty

- >50% off patients treated with lymphodepletion and adoptive cell transfer of autologous tumor infiltrating lymphocytes (TIL) experience objective regression
  - Surgery is necessary
  - Ex vivo identification and explansion of cells

# Early Methods



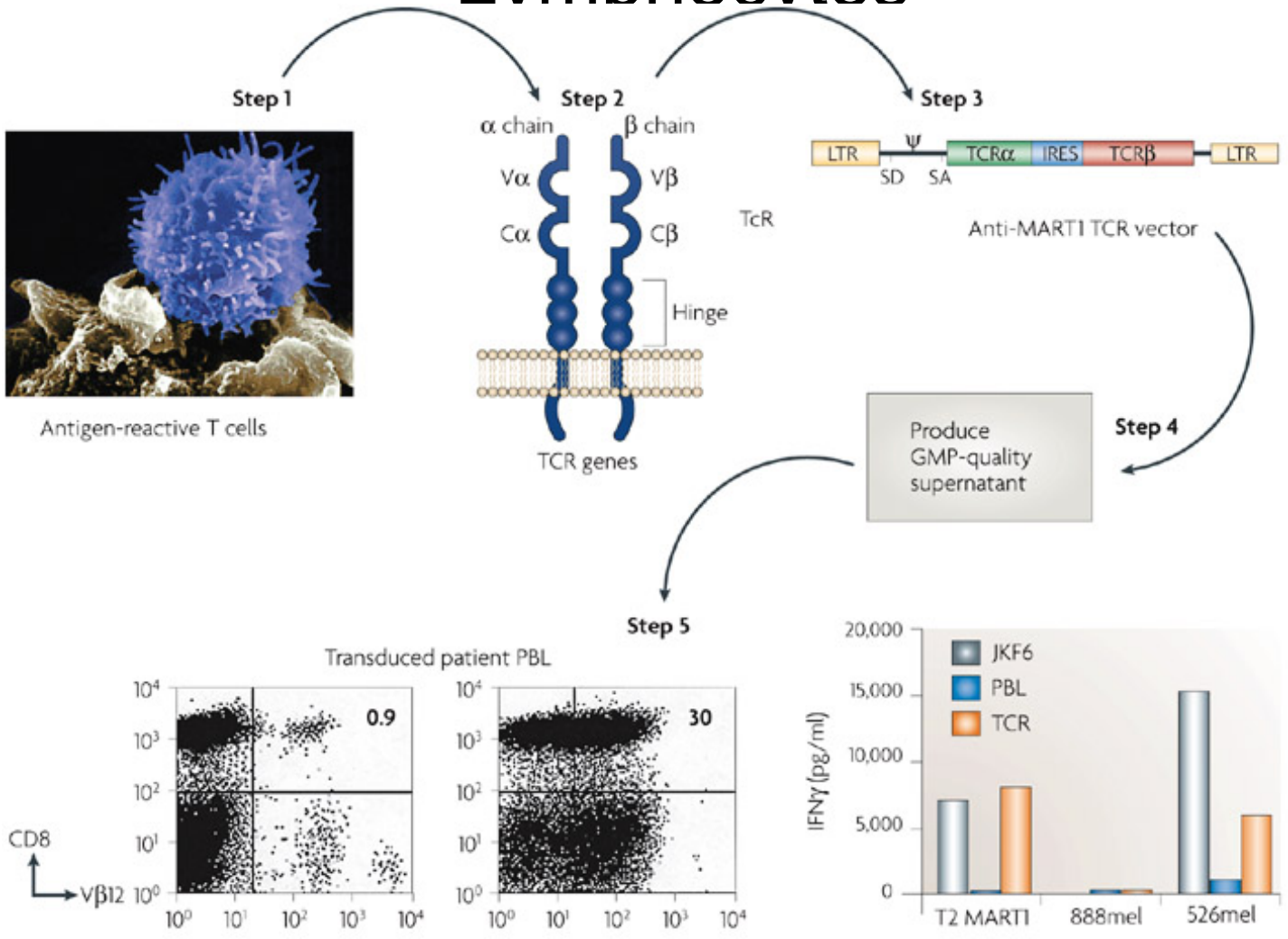



Source: Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer. 2008 Apr;8(4):299-308.

# Lessons Learned

- TCR gene-modified T cells are less effective than TIL
  - Can be activated
  - Can secrete cytokines
  - Can lyse target cells
- Patients must express (HLA)-A*0201 and have tumors that express a common melanoma associated antigen (MAA)

# Later Methods – Gene Modified Lymphocytes



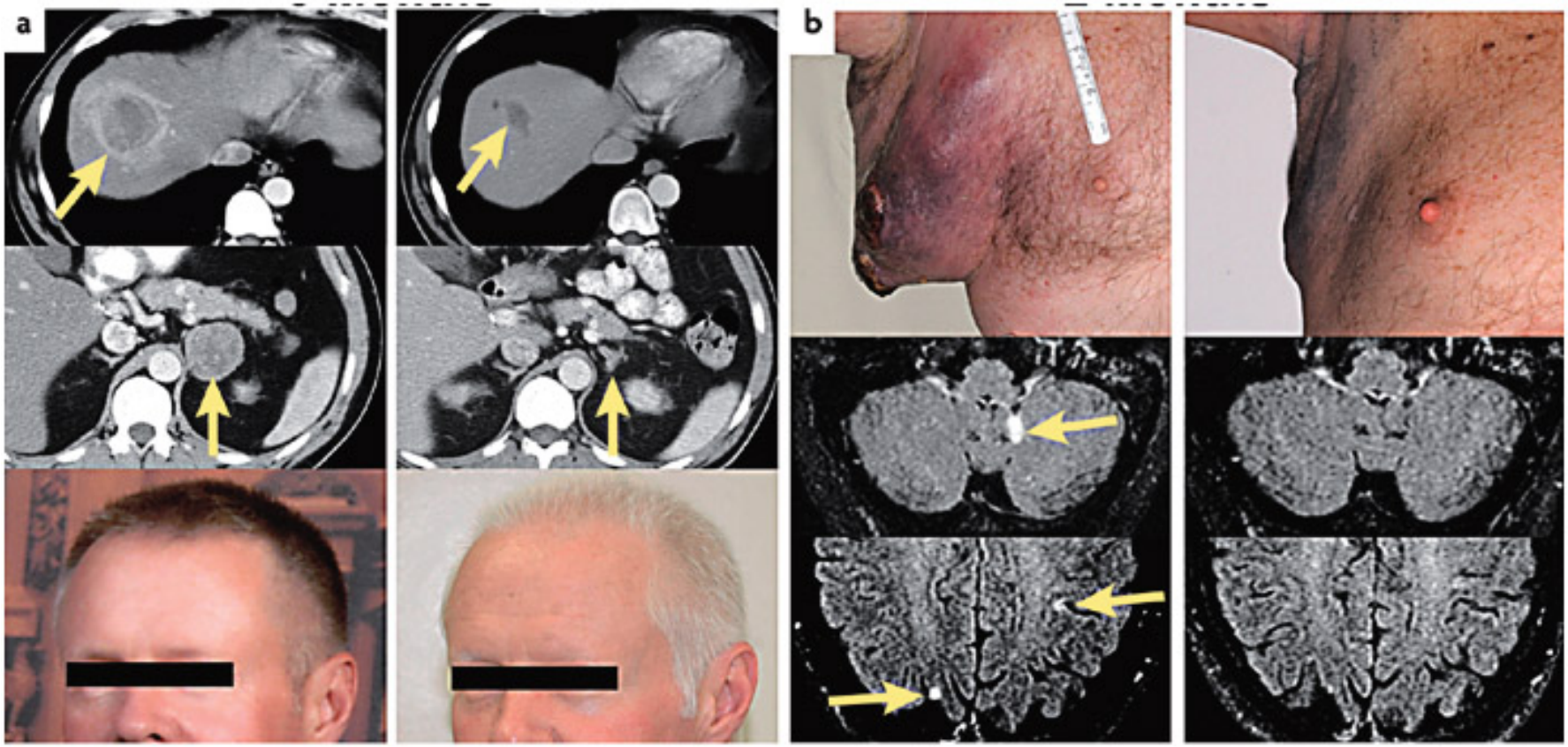



Nature Reviews | Cancer



Source: Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer. 2008 Apr;8(4):299-308.

# Clinical Results






Source: Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer. 2008 Apr;8(4):299-308.

# CHIMERIC ANTIGEN RECEPTORS

# Chimeric Antigen Receptors (CARs)

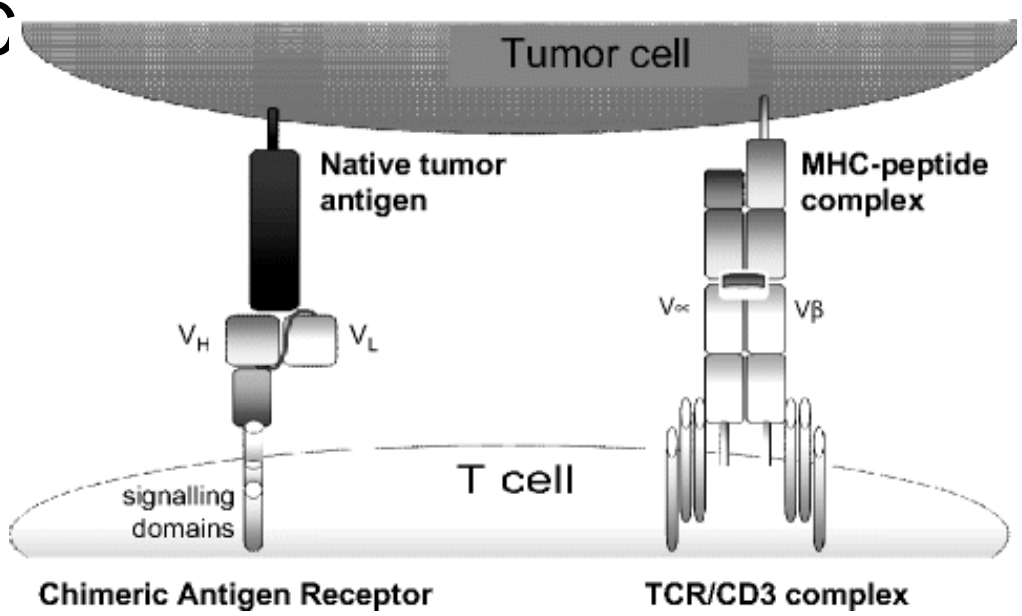
- Consist of:
  - Extracellular antigen recognition domain
  - Intracellular T cell activation domain




Source: Almåsbak H, Lundby M, Rasmussen AM. Non-MHC-dependent redirected T cells against tumor cells. Methods Mol Biol. 2010;629:453-93. Schumacher, Nat Reviews Immunol, 2002

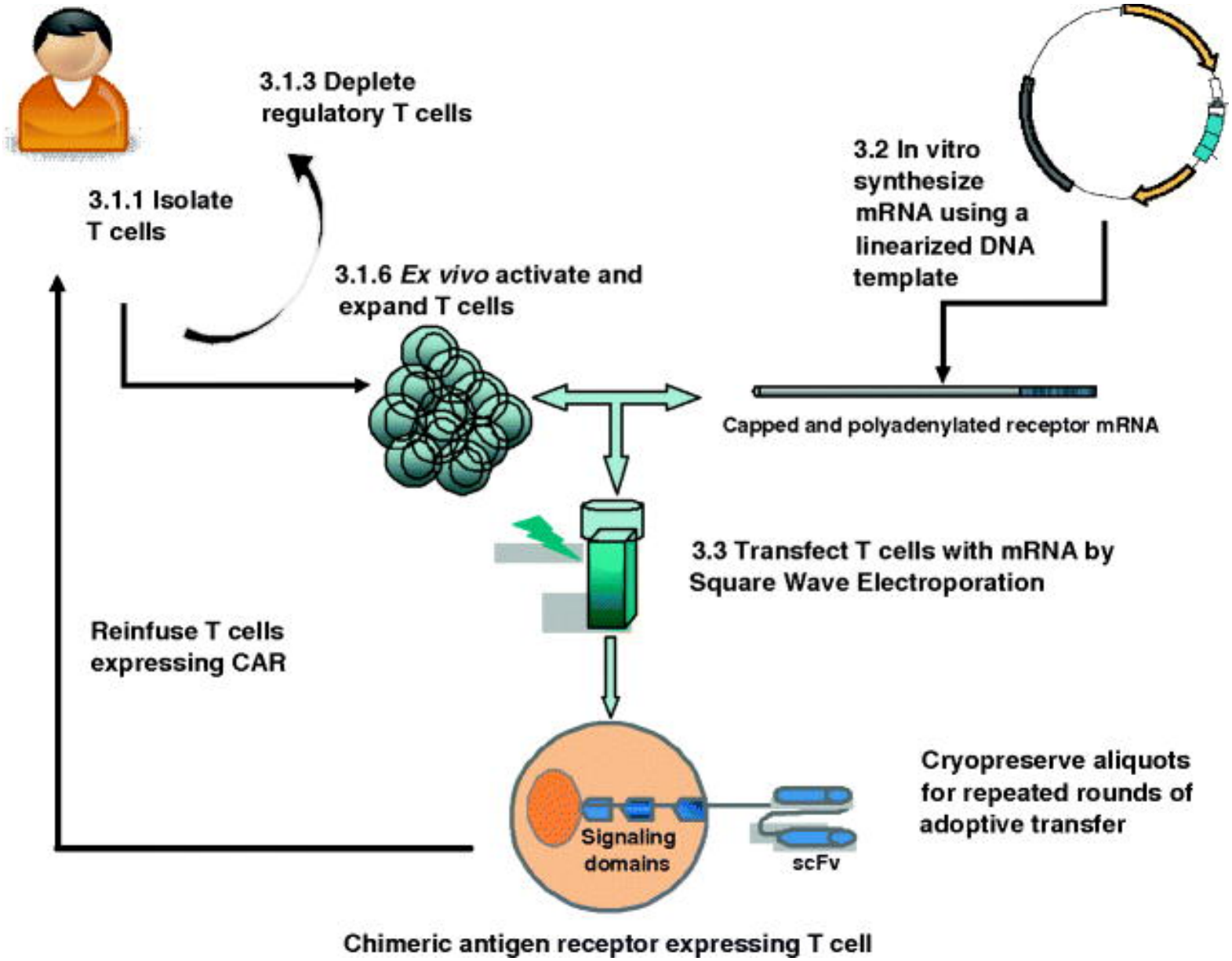
# Rationale

- Not restricted by MHC
  - Antigen recognition domain = Single chain antibody fragment
- No need for
  - dimerization of TCR
  - Antigen processing






Source: Almåsbak H, Lundby M, Rasmussen AM. Non-MHC-dependent redirected T cells against tumor cells. Methods Mol Biol. 2010;629:453-93.





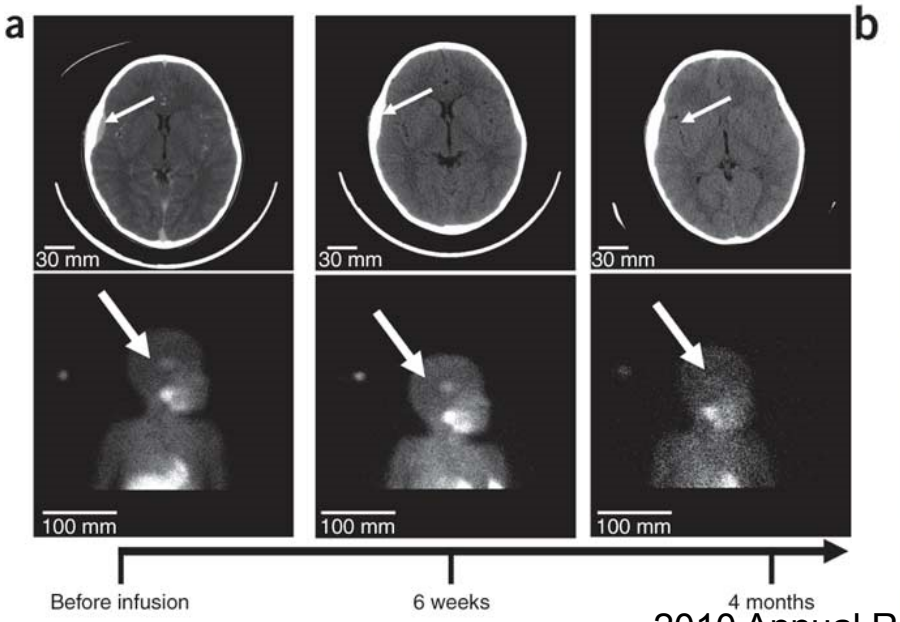

Source: Almåsbak H, Lundby M, Rasmussen AM. Non-MHC-dependent redirected T cells against tumor cells. Methods Mol Biol. 2010;629:453-93.

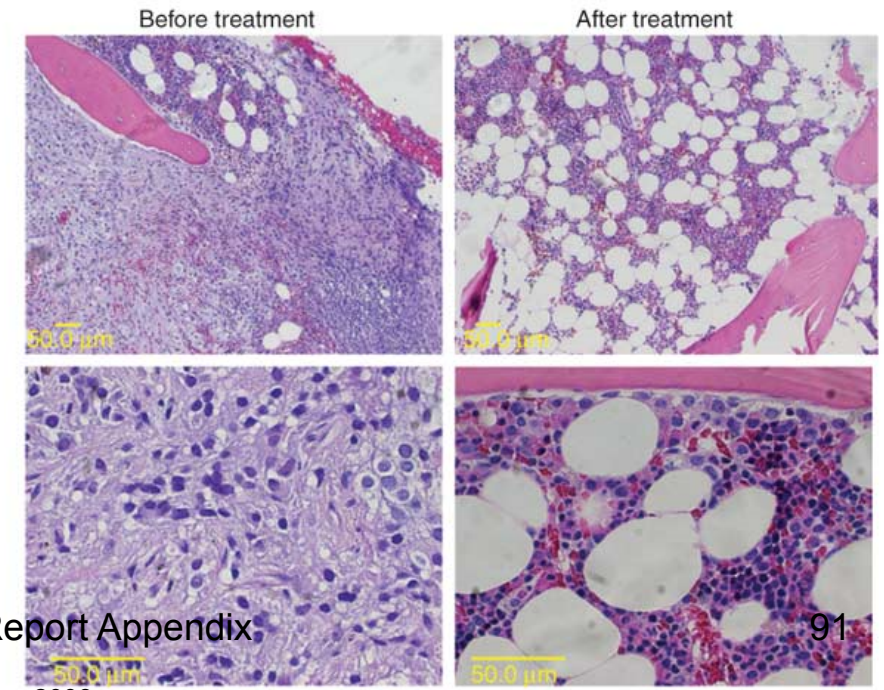
# CARs in Clinical Trials - Neuroblastoma

w.nature.com/naturemedicine

## Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma

Martin A Pule[1], Barbara Savoldo[1], G Doug Myers[1,2], Claudia Rossig[1], Heidi V Russell[1,2], Gianpietro Dotti[1,3], M Helen Huls[1], Enli Liu[1], Adrian P Gee[1–3], Zhuyong Mei[1], Eric Yvon[1], Heidi L Weiss[4], Hao Liu[4], Cliona M Rooney[1,2,5], Helen E Heslop[1–3] & Malcolm K Brenner[1–3]






Pule et al. Nature Medicine 14, 1264 - 1270 (2008) Published online: 2 November 2008

# CARs in Clinical Trials - Neuroblastoma



*original article*

## Adoptive Transfer of Chimeric Antigen Receptor Re-directed Cytolytic T Lymphocyte Clones in Patients with Neuroblastoma

Julie R Park[1], David L DiGiusto[2], Marilyn Slovak[3], Christine Wright[2], Araceli Naranjo[4], Jamie Wagner[4], Hunsar B Meechoovet[4], Cherrilyn Bautista[4], Wen-Chung Chang[4], Julie R Ostberg[4] and Michael C Jensen[5]

| Patient no. (UPN) | Tumor response | | Response to additional therapy[a] | Time to relapse (days after last infusion) | Outcome | Survival (days after first infusion) |
|---|---|---|---|---|---|---|
| | Day 35 | Day 56 | | | | |
| **014** | PD | PD | CR | 42 | DOD | 536 |
| **015** | PD | PD | PD | 0 | DOD | 180 |
| **016** | SD | PR | PD | 174 | DOD | 575 |
| **017** | PD | PD | PD | 0 | DOD | 329 |
| **019** | NE | PD | SD | 35 | DOD | 1,670 |
| **022** | PD | PD | PD | 12 | DOD | 162 |



*Molecular Therapy* (2007) **15** 4, 825–833

# CARs in Clinical Trials – Ovarian Cancer

***Cancer Therapy: Clinical***

## A Phase I Study on Adoptive Immunotherapy Using Gene-Modified T Cells for Ovarian Cancer

Michael H. Kershaw,[1,3,4] Jennifer A. Westwood,[1,3] Linda L. Parker,[1] Gang Wang,[1,5] Zelig Eshhar,[6] Sharon A. Mavroukakis,[1] Donald E. White,[1] John R. Wunderlich,[1] Silvana Canevari,[7] Linda Rogers-Freezer,[1] Clara C. Chen,[2] James C. Yang,[1] Steven A. Rosenberg,[1] and Patrick Hwu[1,5]

T cells with reactivity against the ovarian cancer–associated antigen α-folate receptor (FR) were generated by genetic modification

No reduction in tumor burden was seen in any patient.



Clinical Cancer Research October 2006 12; 6106

# CARs in Clinical Trials – Renal Cell Carcinoma

VOLUME 24 · NUMBER 13 · MAY 1 2006

JOURNAL OF CLINICAL ONCOLOGY

CORRESPONDENCE

## Treatment of Metastatic Renal Cell Carcinoma With Autologous T-Lymphocytes Genetically Retargeted Against Carbonic Anhydrase IX: First Clinical Experience

No reduction in tumor burden was seen in any patient.



*Journal of Clinical Oncology*, Vol 24, No 13 (May 1), 2006: pp. e20-e22

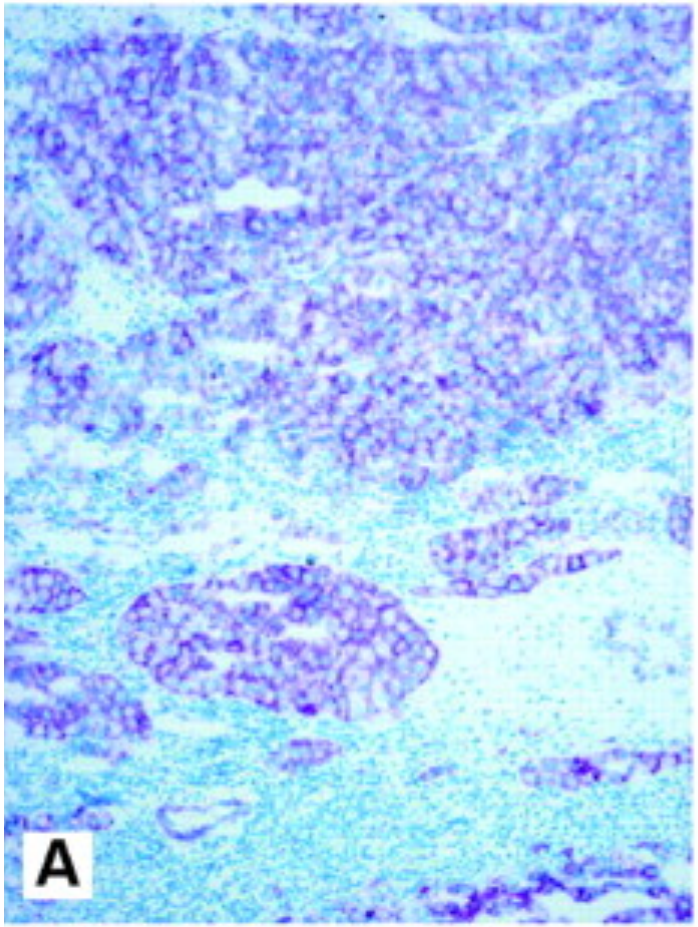
# Goals of this Paper

1. Develop a non-MHC-restricted CAR to target HMW-MAA
2. Optimize construct for use in human PBL
3. Generate a product for use in the clinic

# Picking the Right Antigen

<u>High Molecular Weight – Melanoma Associated Antigen (HMW-MAA)</u>

- Cell surface proteoglycan
- Expressed on >90% of melanomas
- May contribute to malignant phenotype via enhancement of spreading, invasion, and migration
- Found in some adult tissues
  - Hair follicles
  - Basal cells of the epidermic
  - Endothelial cells
  - Activated pericytes




Expression of a HMW-MAA in a metastatic lymph node of a patient with melanoma. Alkaline phosphatase-anti–alkaline phosphatase immunostaining method; hematoxylin counterstain, original magnification, ×100. Source: Clinical Cancer Research June 1, 2006 Vol. 12 no. 11 3297-3305

# Looking for Antigen Expression

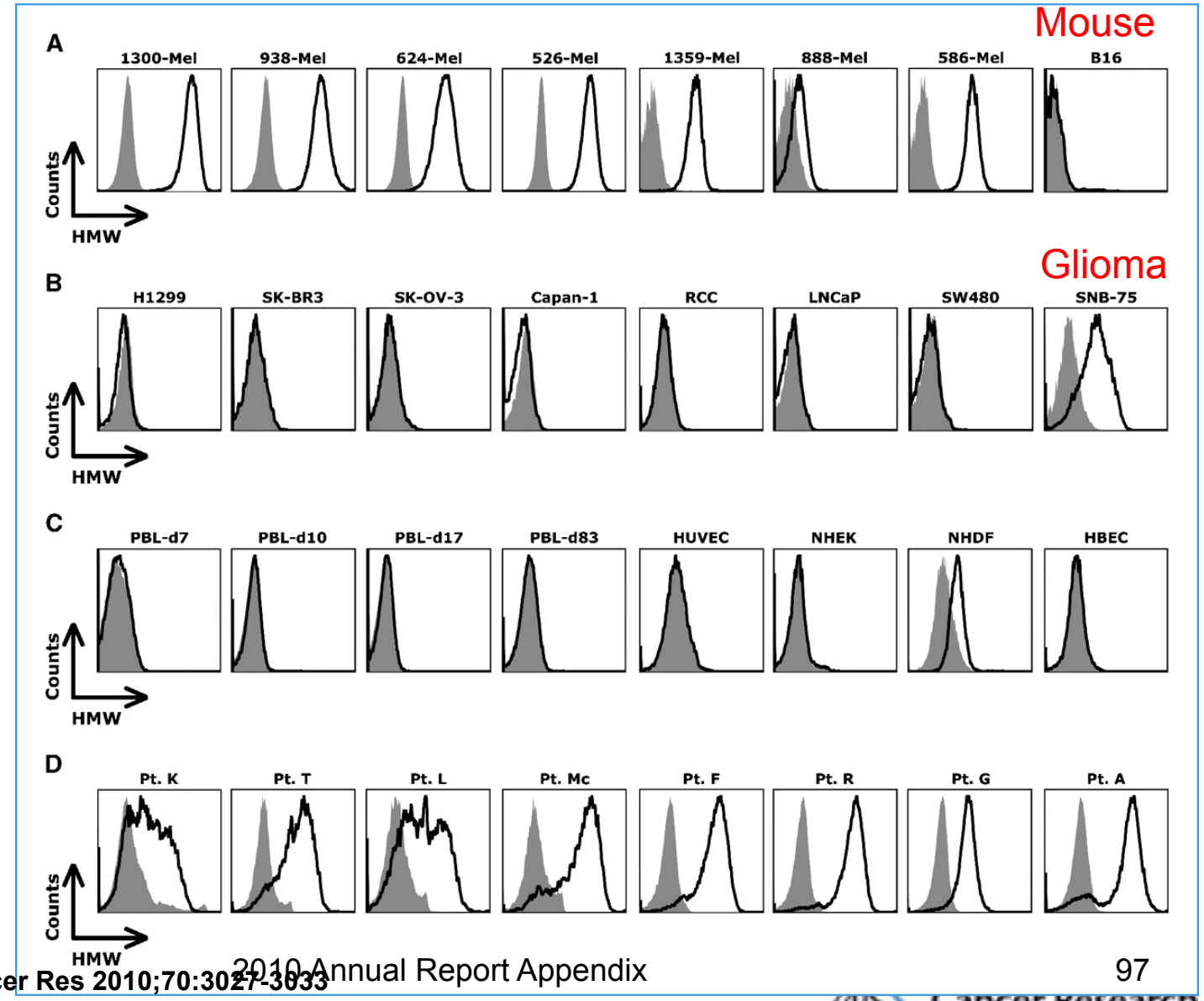
**Figure 1. HMW-MAA was highly expressed on human melanoma lines and explanted melanoma tumors**

Melanoma Cell Lines

Non-Melanoma Cell lines

Ex vivo PBL and normal cell culture

Explanted Tumors





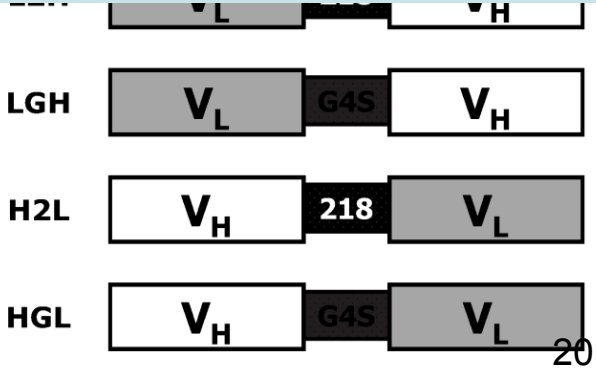

**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**

# Human HMW-specific scFv cassettes

Differed by

- order of light and heavy chain regions
- addition of flexible linker peptides

Tested expression and function of CAR transfected cells after overnight culture with a melanoma cell line




| Cassette | % Transduction | IFN-γ Release pg/ml |
|---|---|---|
| LGH | 40 (12) | 8815 (5809) |
| H2L | 19 (9) | 3808 (2196) |
| HGL | 27 (4) | 6577 (420 |
| | 17 (2) | 7055 *2084) |

# Building a CAR

Cloned into MSGV-1 based retroviral vector containing CD28 and CD3ζ signaling domains

Added 4-1BB (CD137) because it may improve function

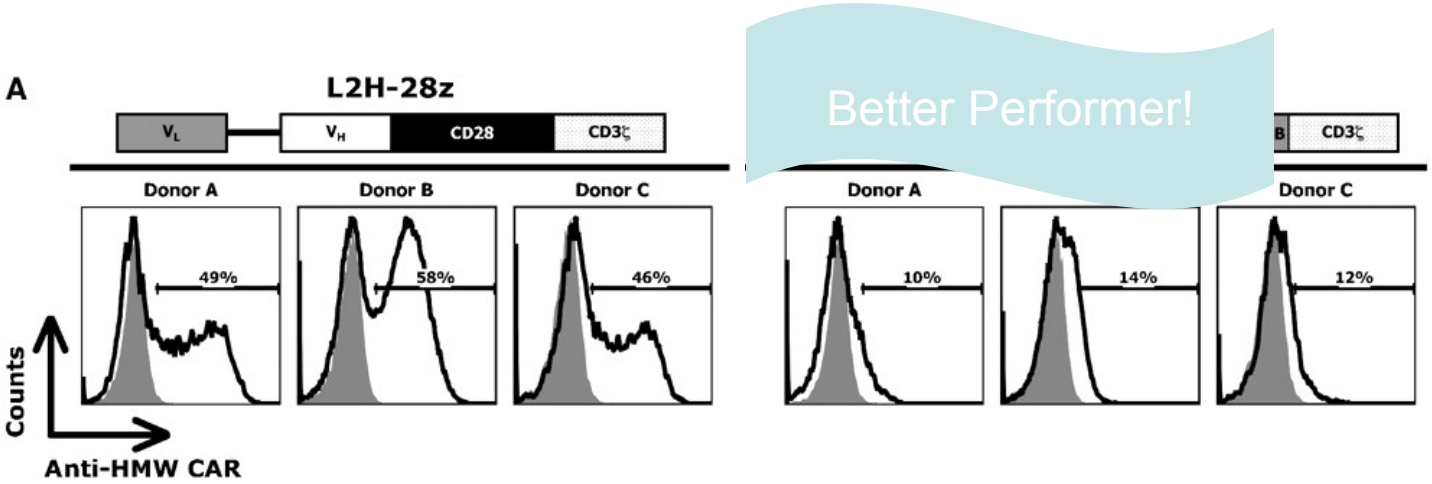
| CAR | % Transduction | IFN-γ Release pg/ml |
|---|---|---|
|  | 53 (6) | 13710 (7134) |
| L2H-28BBz  | 20 (3) | 4732 (2504) |
| L2H-CD8.28BBz  | 14 (1) | 15195 (8255) |

**S3. Anti-HMW CAR constructs with various intracellular domains.** Three anti-HMW CAR constructs were generated using the optimal 225.28S-based scFv (L2H: $V_L$-218-$V_H$) for the extracellular domain and one of three intracellular domains, which differed in their T cell signaling motifs. L2H-28z (containing CD28 transmembrane / signaling domain and CD3ζ

# Comparing Two Constructs

**Figure 2. PBL transduced to express an optimized HMW-MAA-specific CAR were redirected to target HMW-MAA-expressing melanoma lines**

**% Gene Transfer and Expression in Patient T Cells**






**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**

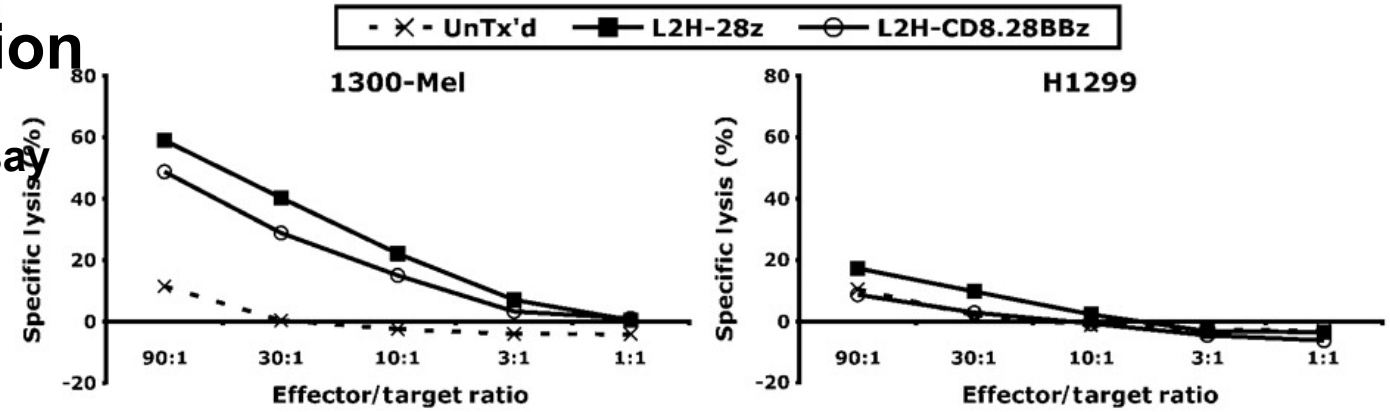
# Comparing Two Constructs

**Figure 2. PBL transduced to express an optimized HMW-MAA-specific CAR were redirected to target HMW-MAA-expressing melanoma lines**


Better Performer!

## Cytolytic Function

### Chromium Release Assay






**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**

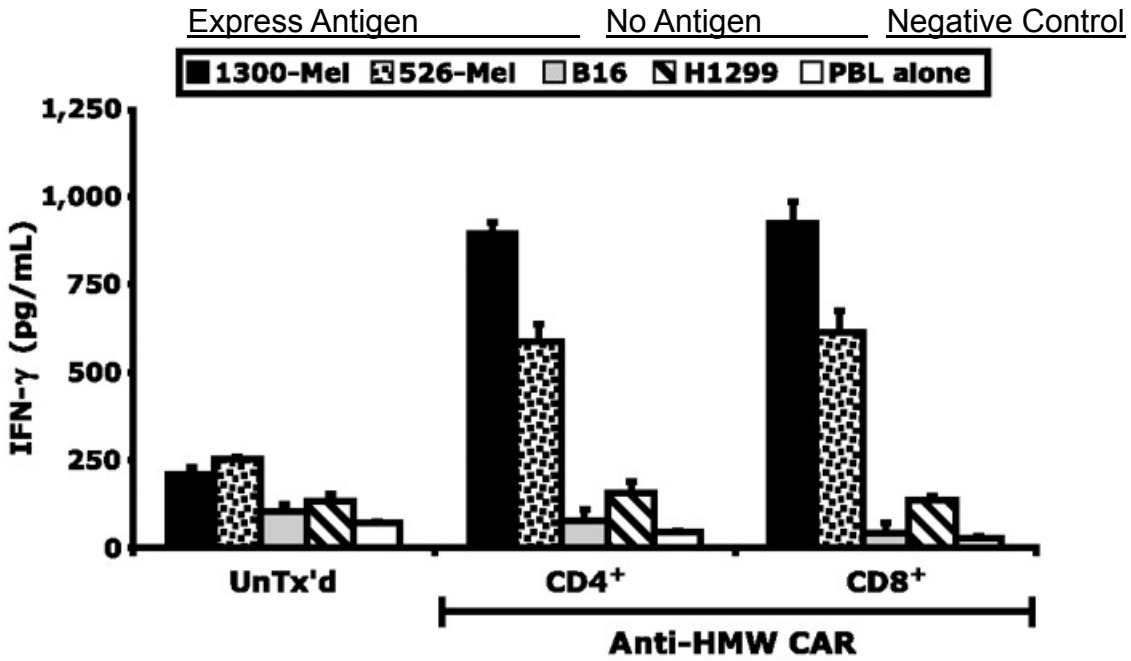


Cancer Research

# Testing CAR Function

**Figure 3. HMW-MAA-specific CAR functions in CD4+ and CD8+ T cells and is non-MHC restricted**

Cytokine release was similar in both cell lines






**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**

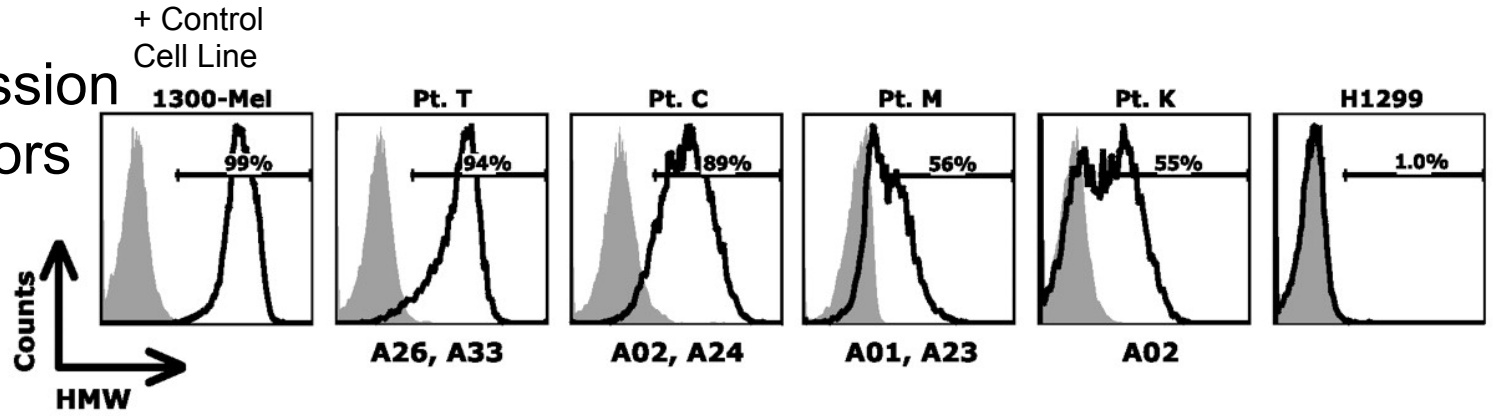


Cancer Research

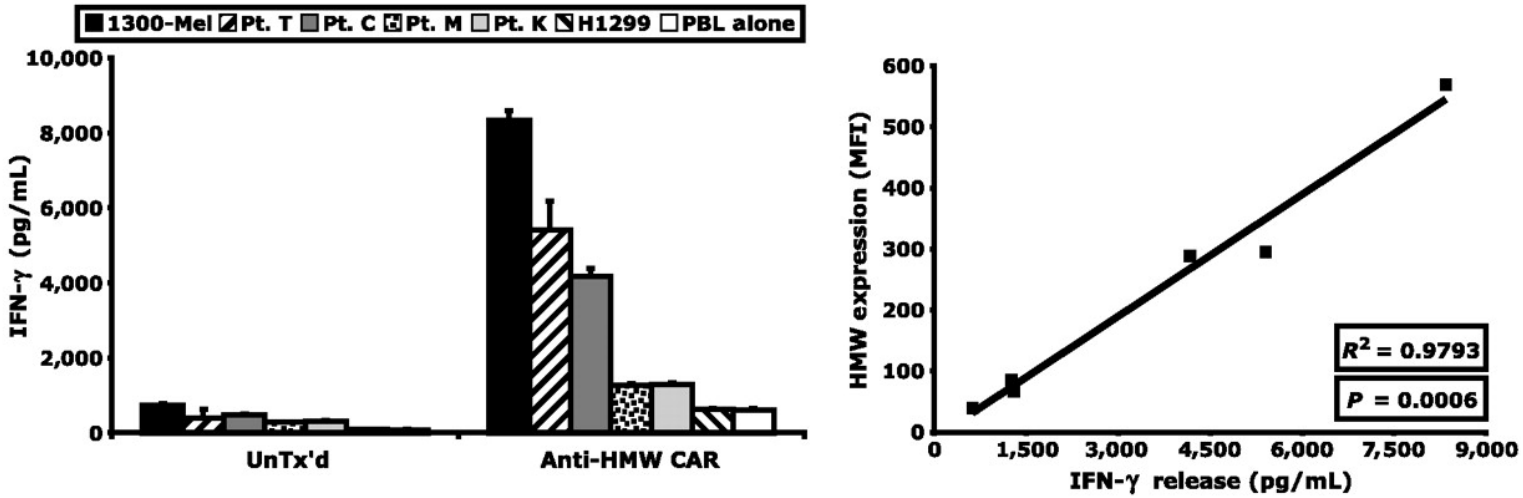
# Testing CAR Function

**Figure 3. HMW-MAA-specific CAR functions in CD4+ and CD8+ T cells and is non-MHC restricted**

Antigen Expression in Patient Tumors




Functional Assays





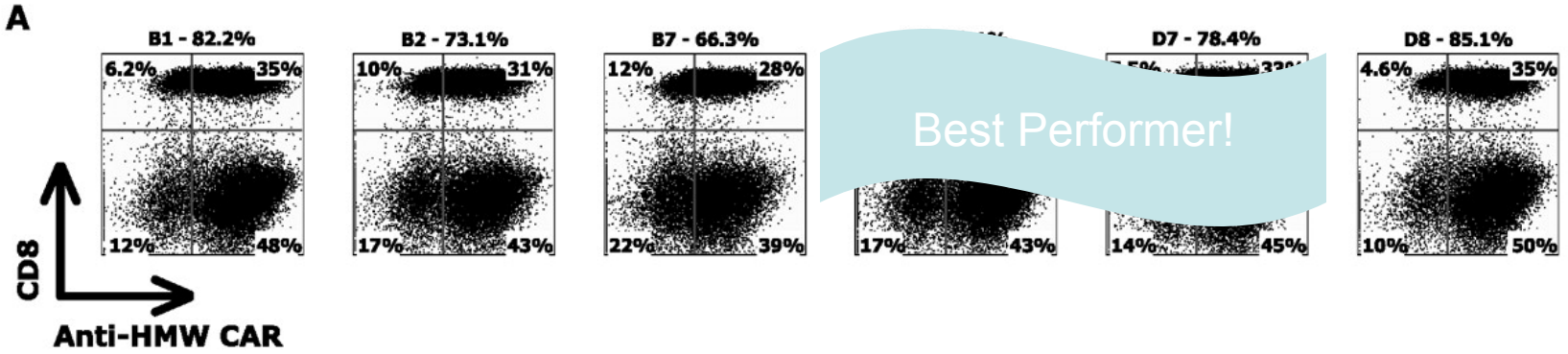

**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**

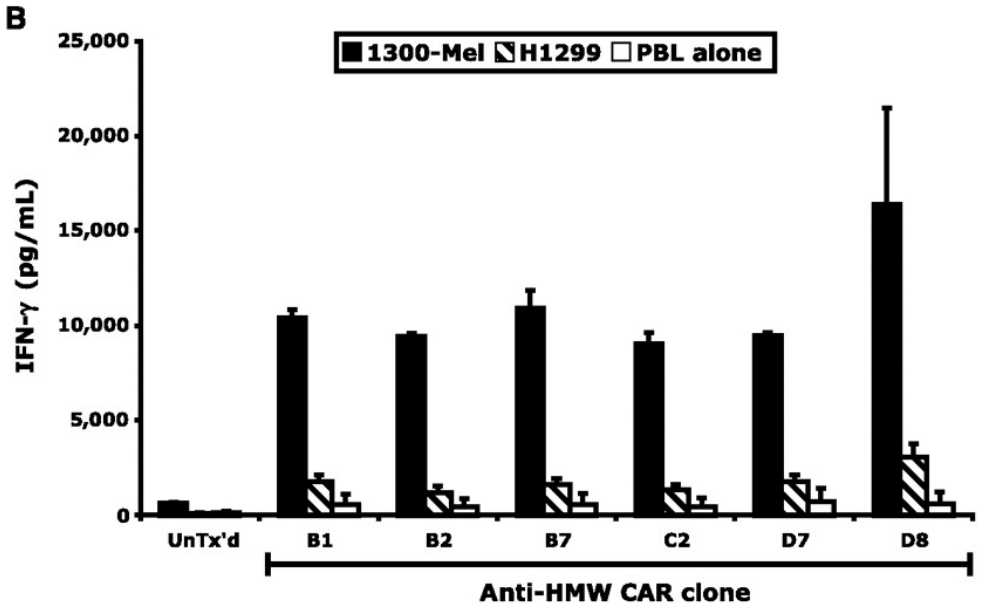


Cancer Research

# Preparing for the Clinic

**Figure 4. A high-titer HMW-MAA-specific CAR retrovirus packaging clone enabled efficient gene transfer and redirected PBL to target a non-HLA-A*02 patient's autologous tumor**






**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**

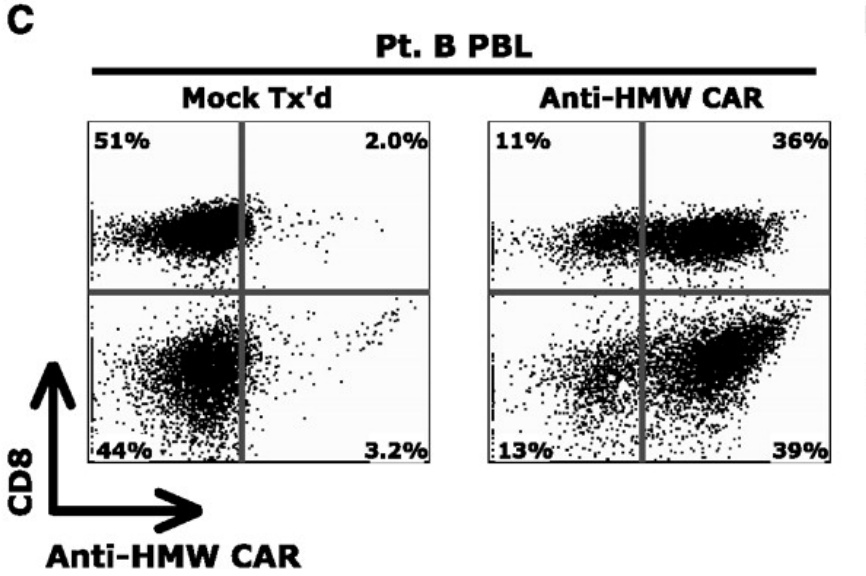


Cancer Research

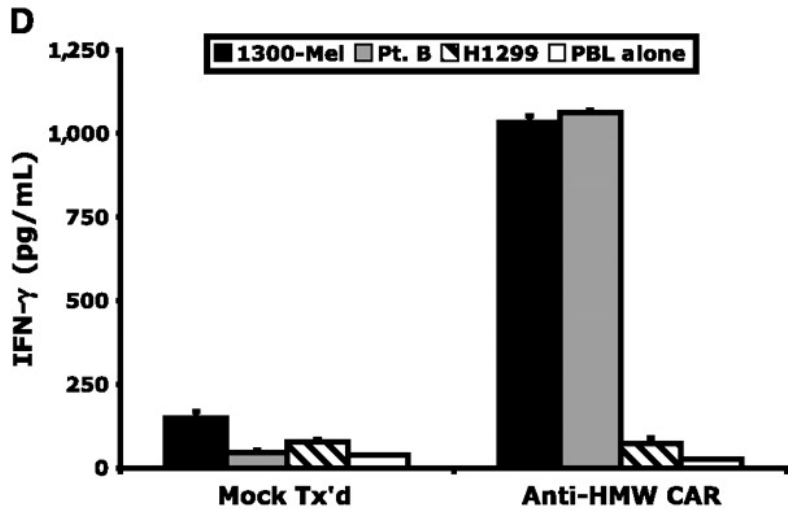
# Preparing for the Clinic

**Figure 4. A high-titer HMW-MAA-specific CAR retrovirus packaging clone enabled efficient gene transfer and redirected PBL to target a non-HLA-A*02 patient's autologous tumor**

## Expression in PBL




## Co-culture with Autologous tumor






**Burns, W. R. et al. Cancer Res 2010;70:3027-3033**



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# Conclusions

- HMW-MAA is expressed on the surface of melanoma cell lines and patient tumors
- An HMW-MAA-specific CAR was created and optimized for expression and function in human T cells and then tested for cytokine secretion, cytolytic activity, and proliferation in response to cells expressing the corresponding antigen
- The optimized HMW-MAA-specific CAR can recognize resected melanomas and was inserted into a CAR retrovirus for clinical use

# Discussion

- Other CARs against the same antigen don’t function
- HMW-MAA is on the cell surface and has a restricted pattern
- Patients need documents expression to receive therapy


Questions?

# Characterization of tumor associated antigens using a recombinant expression of a patient derived breast cancer cDNA library and autologous serum

Leah Novinger
Krag Laboratory
MD/PhD Research Day
Friday, July 23rd, 2010

# Overview

- Background and Rationale
- Methods for cDNA Library Synthesis and Recombinant Expression
- Results
- Future Directions

# Background –SEREX

- SEREX is the serological analysis of recombinant cDNA expression libraries of human tumors with autologous serum
- Concept: Insert mRNA for tumor proteins into lambda phage genome and express proteins in bacteria, then identify antibodies that bind to these proteins
- Rationale: Antigen identification may aid in diagnosis, treatment, and prognosis of cancer. Antibodies to intracellular and extracellular antigens may be identified and often correlate with a cell mediated response.

# Background

- SEREX will identify:
  - unusual proteins not found in differentiated tissues
  - mutated proteins
  - out of frame sequences
- SEREX will not identify post-translational modifications
- Applications:
  - Diagnostics
  - Measuring treatment response
  - Vaccines
  - Antibody Therapies

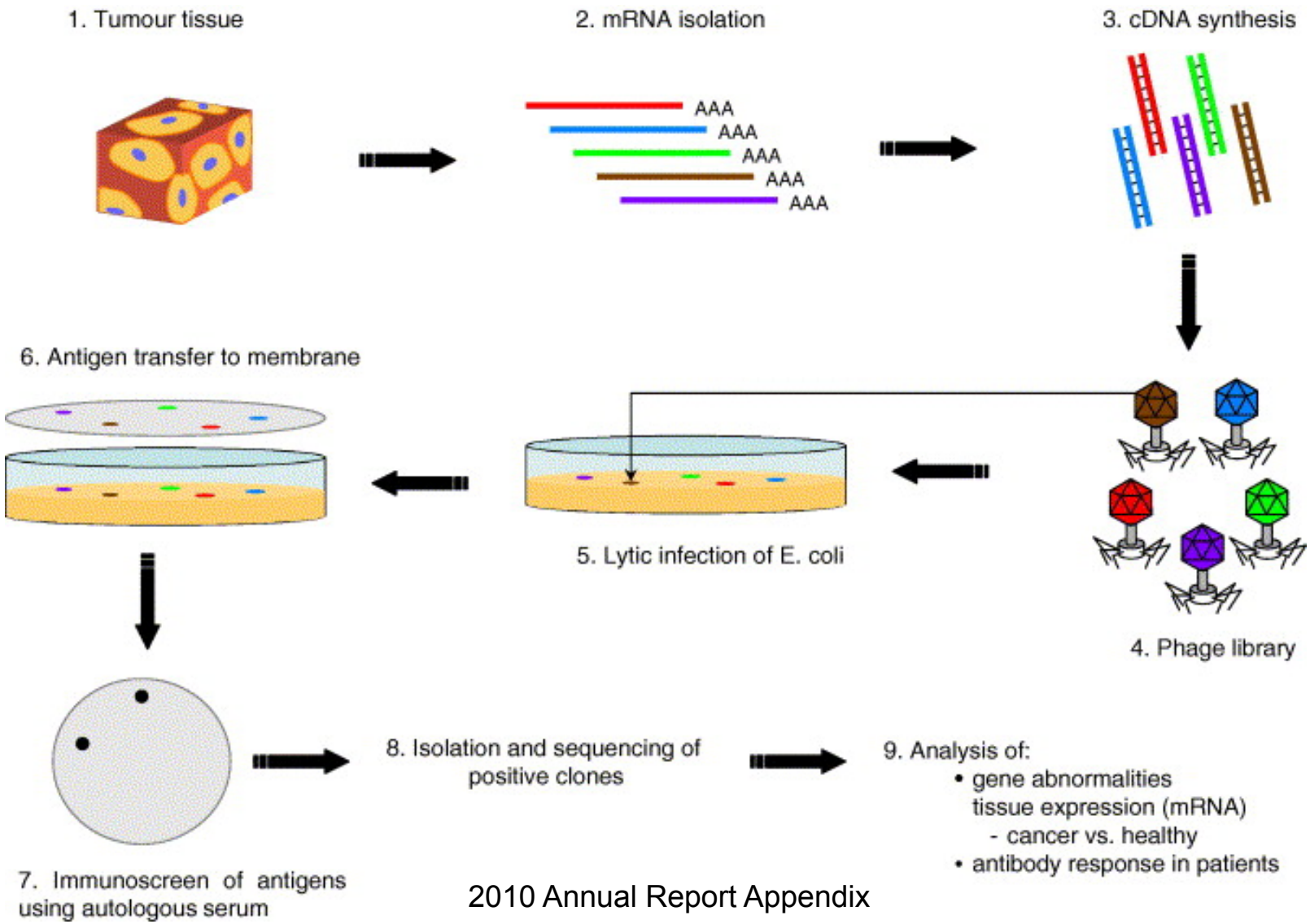
# Research Questions

What kind of antigens are expressed on the patient's tumor?

What is the antibody profile against tumor associated antigens in different tissues?

Hypothesis: Breast cancer patients develop antibodies to specific tumor associated antigens associated with no or very low expression in normal tissues

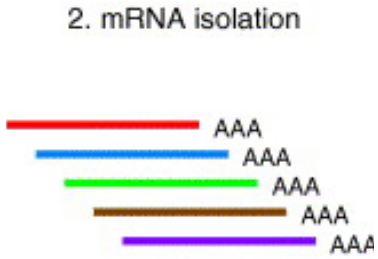
# SEREX

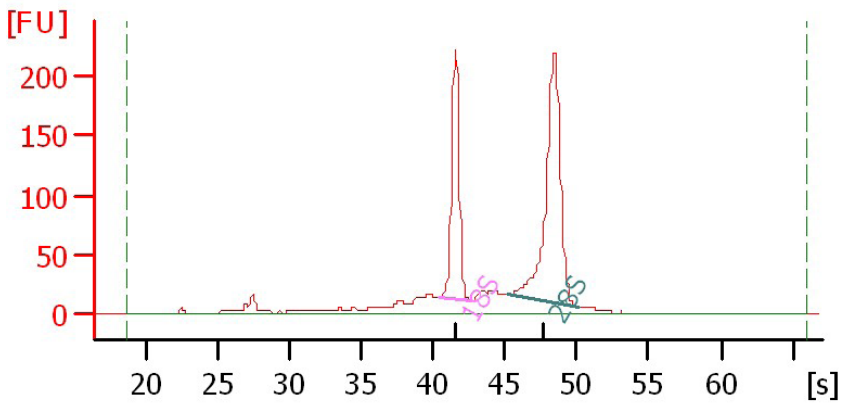




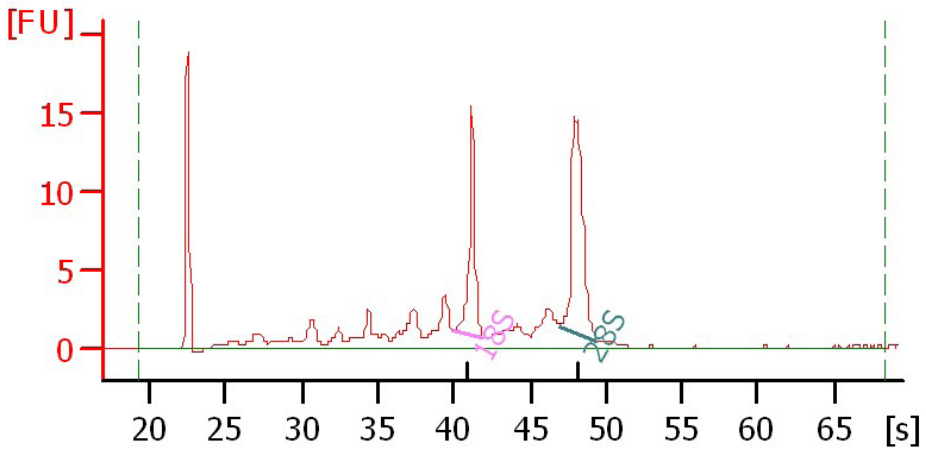

Adapted From Cancer Lett. 2007 Apr 28;249(1):110-9

# Results: Total RNA Isolation




**Cell Line RNA (SkBr3)**

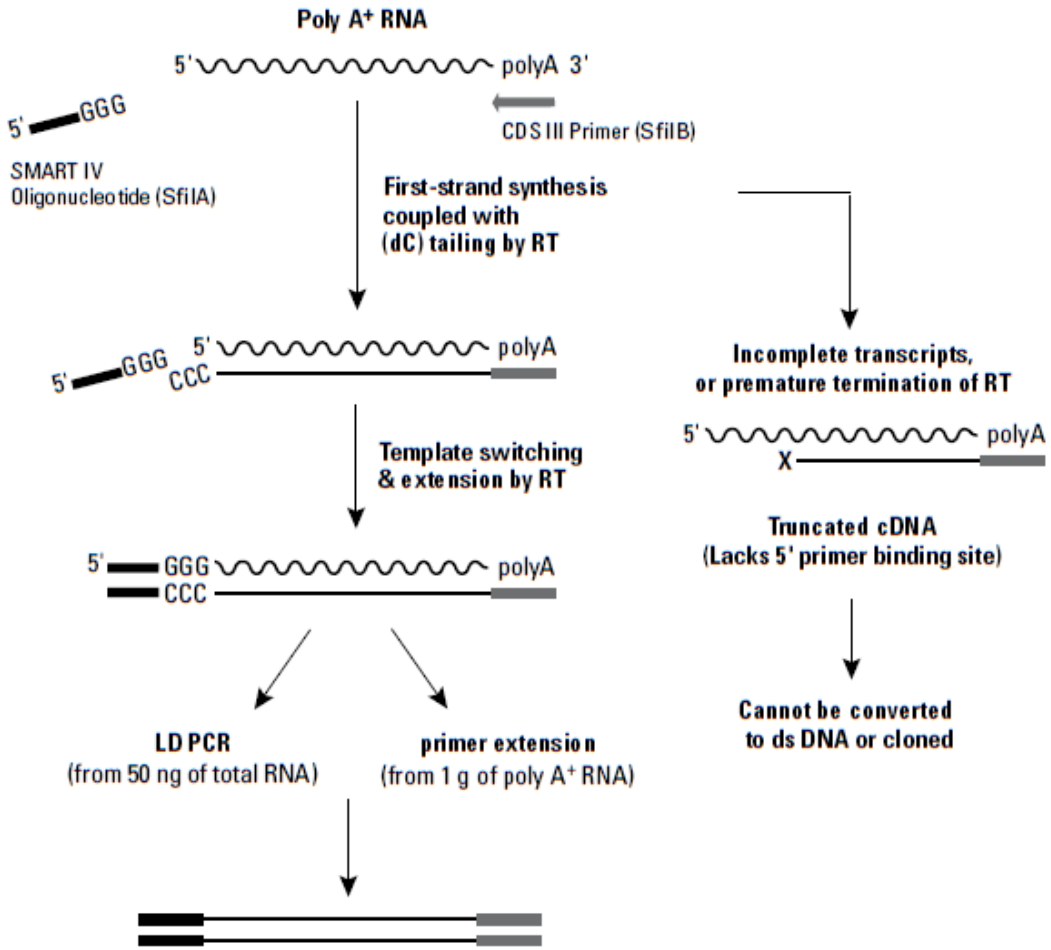



**(09-067-1)**

SkBr3 Sample 14, combined with others to make SkBr3 – 4 mRNA

09-067-1 tumor 1 total RNA

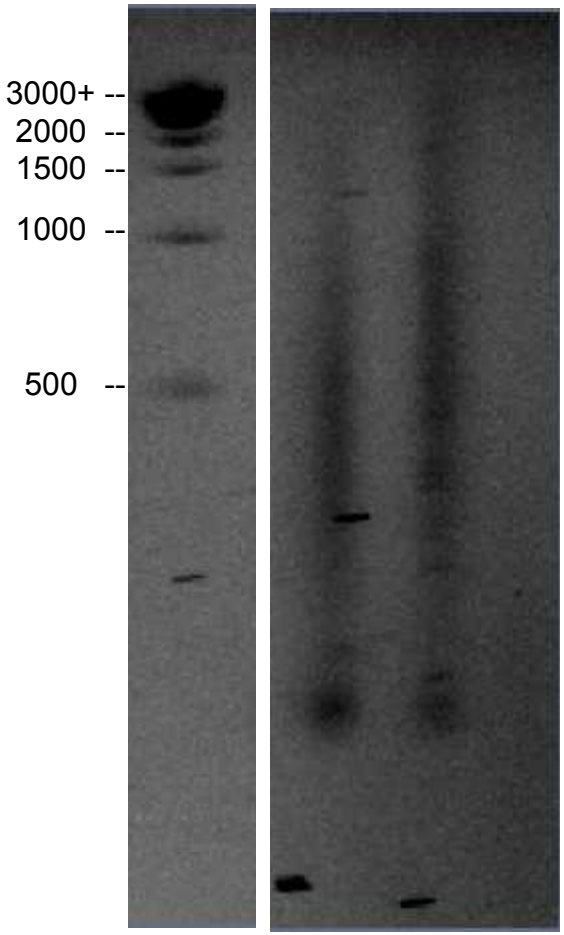
# cDNA Synthesis



# Results: cDNA Library Synthesis

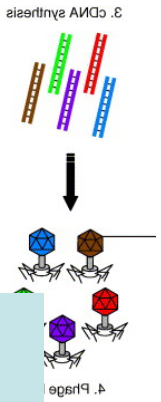

SkBr 3 Library

Ladder 1st strand 2nd strand



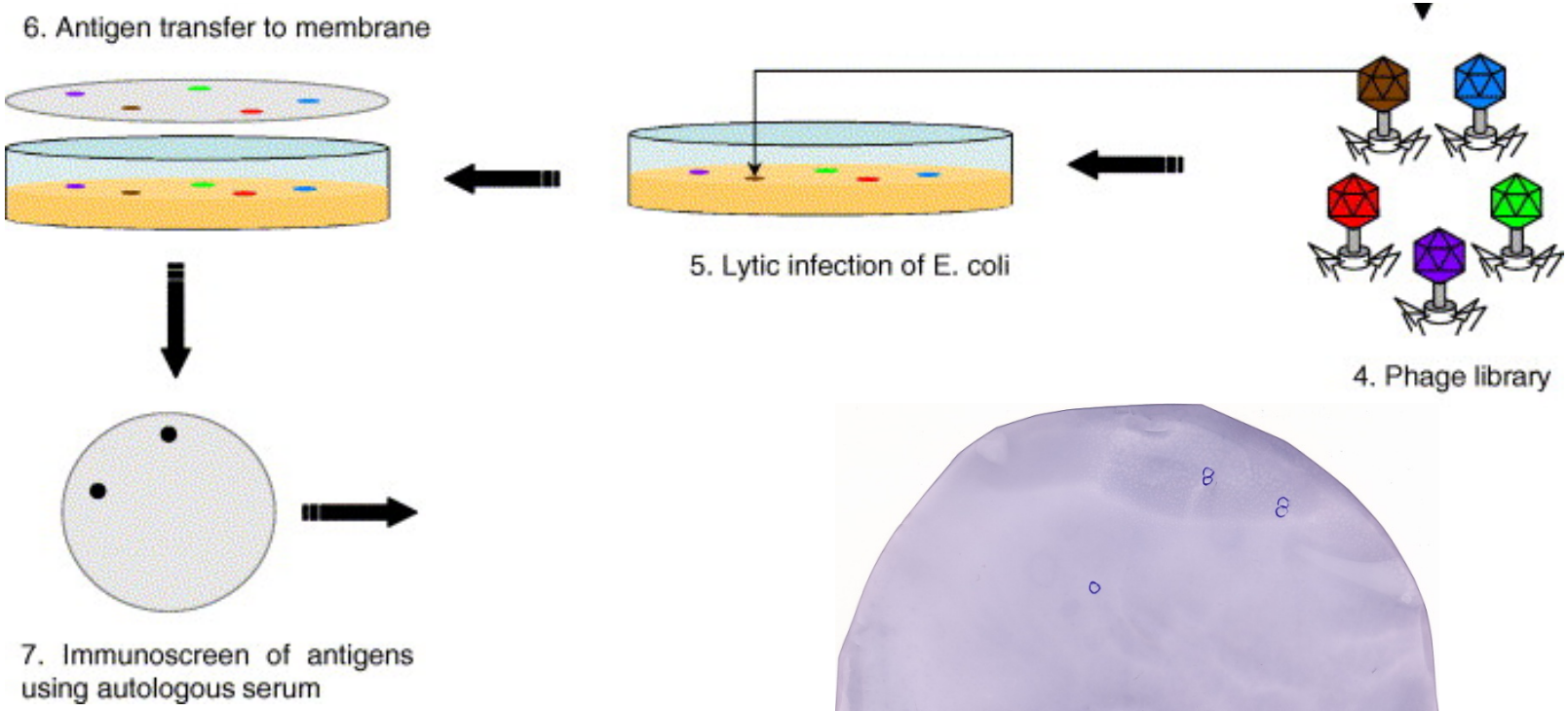

188-6 Tumor Library – ds cDNA Fra

# Results: cDNA Library Evaluation



| Library | Source | Titer | Recombinant |
|---|---|---|---|
| SkBr3 | Cell line | U: **$8.92x10^5$**<br>A: $2.3x10^9$ | >90% |
| 188-6 | Fresh frozen tumor | U: **$8.78x10^5$**<br>A: $5.6x10^7$ | 86 – 96% |
| 09-067-1 | Fresh frozen tumor | U: **$1.26x10^6$**<br>A: $6.85x10^8$ | >90%? |

# Results: Immunoscreening



# Positive Clone Analysis

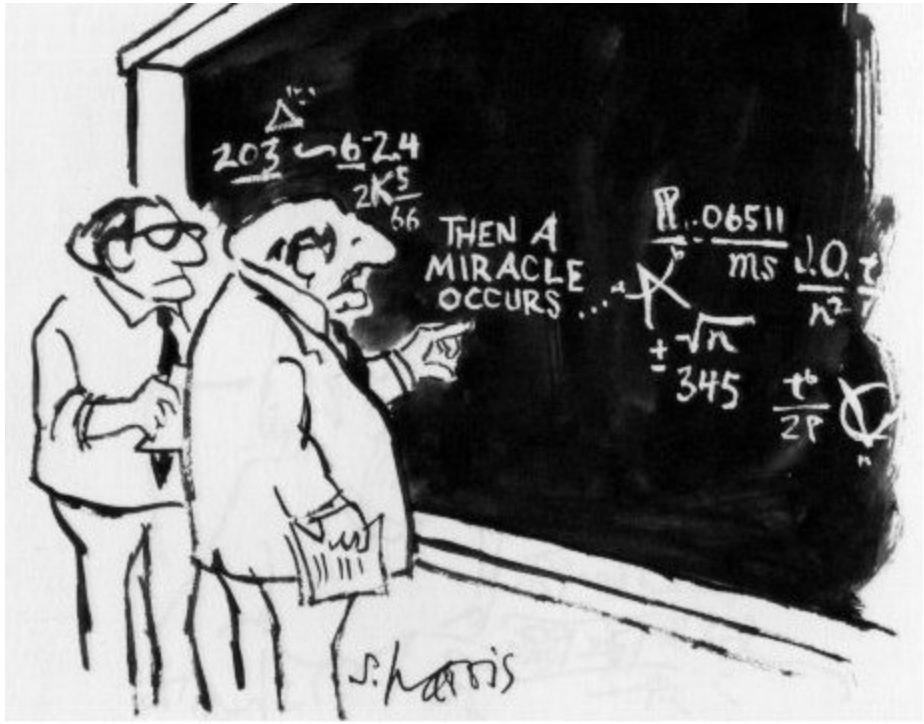
- Secondary Immunoscreening
- Sequencing and Identification

- Number of positive clones expected: <100
- Type of tumor antigens expected
  - Cancer testis antigens
  - Open reading frames (ORFs)
  - Mutated proteins
- False positives
  - IgG encoding cDNAs

# Next Steps

- Continue primary and secondary screening
- Evaluate presence of positive clones in tumor and normal tissues

- Develop antibody libraries examining the patient's antibody repertoire using different tissue sources
  - Tumor
  - Draining lymph nodes
  - Blood




"I think you should be more explicit here in step two."

from *What's so Funny about Science?* by Sidney Harris (1977)

Source: http://www.tc3.edu/instruct/sbrown/stat/showwork.htm

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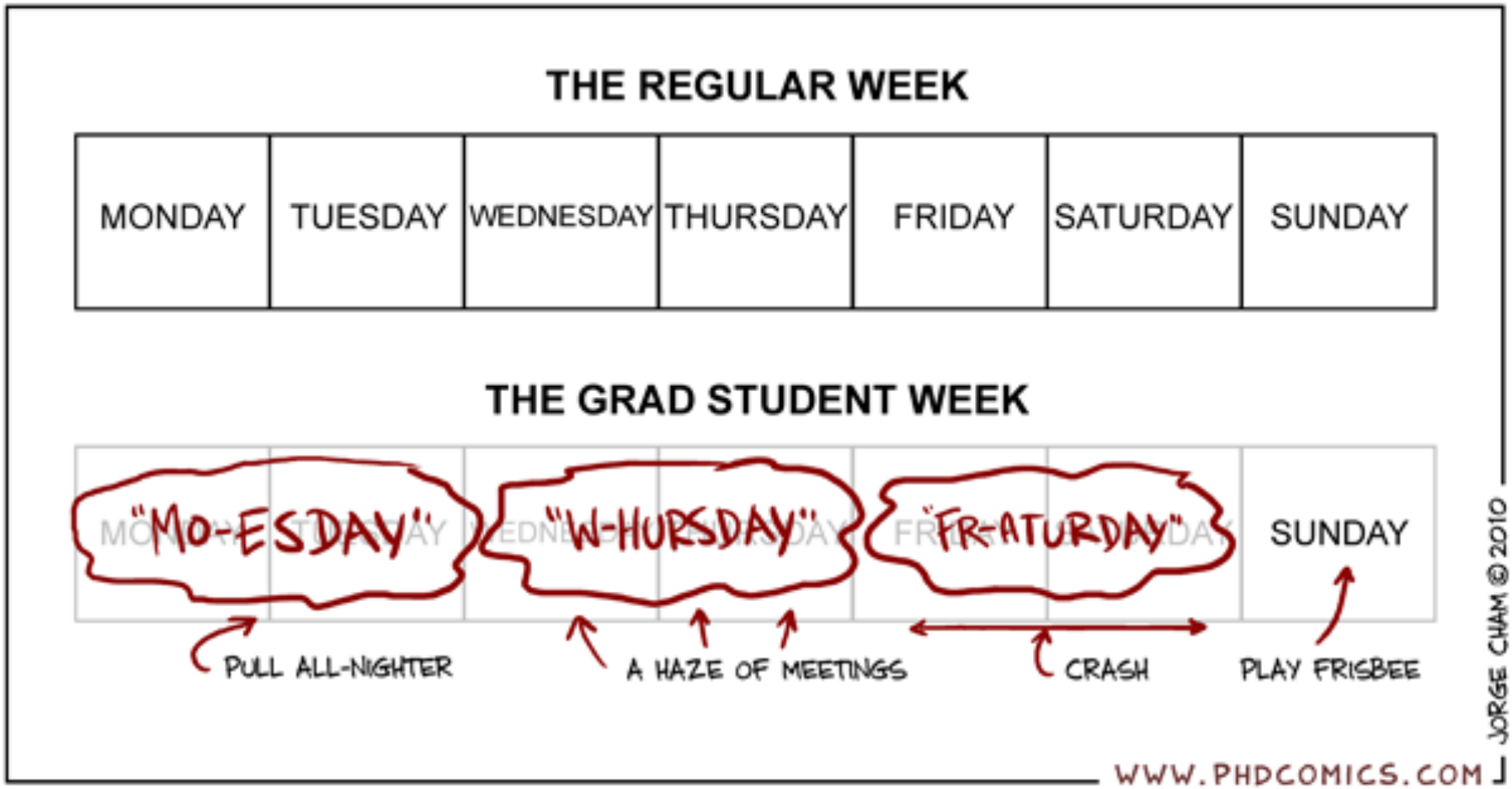
# Questions?


THE REGULAR WEEK
MONDAY
TUESDAY
WEDNESDAY
THURSDAY
FRIDAY
SATURDAY
SUNDAY
THE GRAD STUDENT WEEK
"MO-ESDAY"
"W-HURSDAY"
"FR-ATURDAY"
SUNDAY
PULL ALL-NIGHTER
A HAZE OF MEETINGS
CRASH
PLAY FRISBEE
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